MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, June 1997, p. 212–238 Vol. 61, No. 2
1092-2172/97/$04.00+0

Stable DNA Replication: Interplay between DNA Replication, Homologous Recombination, and Transcription

TOKIO KOGOMA*

Departments of Cell Biology and Microbiology and Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131

* Mailing address: Department of Cell Biology, Rm. 217, Cancer Center, University of New Mexico School of Medicine, Albuquerque, NM 87131. Phone: (505) 272-8825. Fax: (505) 272-6943. E-mail: kogoma@medusa.unm.edu.

INTRODUCTION

> "Oh, you are going to talk about the effects of banging on a network!"
>
> Sydney Brenner (151)

Evolution allows room for redundancy in vital functions. *Escherichia coli* cells possess, in addition to the normal mode, at least three alternative modes of chromosome replication. These modes of replication differ from each other, in essence, in the manner by which they achieve local duplex opening, which is a prerequisite to replication fork assembly. The initial duplex opening that occurs at the normal origin of chromosome replication, *oriC*, is accomplished by binding of the initiator protein, DnaA, to the 9-bp repeats (DnaA boxes) clustered within the *oriC* site. In SOS-induced cells, strand separation can occur by formation of a D-loop, an intermediate in homologous recombination. In yet another circumstance, strand separation can be induced by hybridizing an RNA transcript to the coding strand displacing the other DNA strand. The resulting structure is an R-loop, which, when stabilized, can become an origin of replication.

One of the legacies of the Copenhagen group led by Ole Maaløe is the proposal that "protein and/or RNA synthesis is required to initiate but not to sustain DNA replication" (152). Thus, addition of chloramphenicol to an exponentially growing culture or starvation for required amino acids results in the cessation of initiation of a new round of chromosome replication, although the round of replication already under way can be completed. Subsequently, this requirement for protein and RNA synthesis was shown to be a unique property of DnaA-dependent initiation at *oriC* (248). The biochemical nature of this requirement for concomitant protein synthesis is not understood (but see the end of this review). It suggests that one or more of the factors essential for the initiation reaction at *oriC* is "unstable" and must be replenished for each new round of initiation. When the condition under which this requirement was circumvented was discovered, the term "stable DNA replication" (SDR) was coined to describe the capacity to undergo chromosome replication in the absence of concomitant protein synthesis (117, 118). The condition that endows cells with the capacity for SDR is the induction of the SOS response (122). Thus, SDR is normally repressed but can be activated by SOS induction; it is designated "inducible stable DNA replication" (iSDR). In attempts to gain some insights into the genetic basis of this activity, mutants that constitutively express an SDR activity were isolated (105, 140). One type of mutation that conferred this phenotype was that in the *rnhA* gene encoding RNase HI, an RNase specific to RNA in the RNA-DNA hybrid form (80, 182). This activity in *rnhA* mutants was designated "constitutive stable DNA replication" (cSDR) to distinguish it from the inducible activity (iSDR). Subsequent studies have revealed that iSDR and cSDR are, despite the superficial similarities, distinct activities arising from two different mechanisms of initiation, which both require no concomitant protein synthesis. More recently, wild-type *E. coli* cells were shown to exhibit an SDR activity without SOS induction (77). The activity, termed nSDR, transiently appears in rapidly growing cells upon entry into the stationary phase.

iSDR is a form of recombination-dependent replication (4, 8). Evidence indicates that a replication activity very similar to iSDR is required for homologous recombination and double-strand break (DSB) repair (109, 114). Evidence also suggests that iSDR might play a crucial role in adaptive mutation (60, 73). cSDR activated in *rnhA* mutants can compensate for the lack of chromosome replication from *oriC*. Thus, *rnhA* mutants can survive complete inactivation of the *dnaA* gene or deletion of the *oriC* site (124). nSDR, which perhaps is mechanistically similar to cSDR, may play important roles in the survival of stationary phase cells. The salient features of *oriC* and these alternative replication systems are compared in Table 1.

In this article, I describe the characteristics of these alternative replication forms and review the evidence that has led to the formulation of the proposed models for SDR initiation mechanisms. I attempt to shed light on the interplay between DNA replication, homologous recombination, DSB repair, and transcription in *E. coli* cells. Brief, condensed reviews of SDR

TABLE 1. Comparison of the replication systems of *E. coli*

Replication system	Type of cells	Mode of duplex opening	Origin used	Requirement for:					
				DnaA	RecA	RecBC	PriA	Protein synthesis	RNA synthesis
DnaA/*oriC*	Normal	DnaA-*ori* interaction	*oriC*	+	−	−	−	+	+
iSDR	SOS induced	D-loop	*oriM* sites	−	+	+	+	−	−
cSDR	*rnhA recG* mutants	R-loop	*oriK* sites	−	+	−	+	−	+
nSDR	Upshifted	R-loop?	*oriK* sites?	−	+	−	?	−	±

and related subjects have already been presented (6, 109). The readers are referred to excellent comprehensive reviews by Messer and Weigel (168) for *oriC* initiation, by Marians (156) for replication fork structures and functions, and by Kowalczykowski et al. (127) and Lloyd and Low (145) for homologous recombination and to a monograph by Kornberg and Baker (126) for general properties of *E. coli* and other DNA replication systems.

INDUCIBLE STABLE DNA REPLICATION

Inducing Conditions

All conditions that induce iSDR also induce the SOS response; these including thymine starvation, UV irradiation, incubation of *dnaB*(Ts) mutants at the restrictive temperature, and exposure to genotoxic agents such as mitomycin, methyl methanesulfonate, and nalidixic acid (117, 122, 138, 162). Like other SOS functions, iSDR induction is blocked by a *lexA* (Ind^-) mutation (122) and rendered temperature sensitive by *recA*(Ts) (138). *recA*(Prt^c) mutations, which cause constitutive activation of RecA coprotease and thereby chronic derepression of the SOS regulon, do not lead to constitutive expression of iSDR (122, 257). This is because iSDR requires an activated form of RecA protein (RecA*) for the activity (see the section on Possible Roles of iSDR, below).

Mode of Replication

After SOS induction, semiconservative DNA replication can continue in the presence of chloramphenicol for many hours; a 16-fold increase in DNA over 20 h has been recorded (117). iSDR can also occur in the presence of rifampin (136). Thus, persisting replication, once it is induced, requires neither translation nor transcription. Since chloramphenicol inhibits the cell mass increase and cell division, a period of iSDR results in cells that are packed with DNA (118). The continued DNA synthesis is not an amplification of selected sequences but a replication of the entire chromosome sequence. Density shift experiments indicated that only a part of the chromosome population is engaged in replication at any time during iSDR (117). These are chosen for templates at random from the pool of accumulating chromosomes. The observed random selection of chromosomes for replication indicates the lack of preference either for the most recently replicated chromosomes or for old chromosomes and rules out the rolling-circle mode of replication as the major mechanism of iSDR. In the rolling-circle mode, one strand is being synthesized on the circle and the other is being synthesized on the strand that has just been displaced from the circle and hence is the most recently replicated DNA (126). The mechanism of iSDR most probably involves a bidirectional θ mode of replication (see the section on Evidence for the D-Loop Model, below).

Origin Usage in iSDR

Density shift experiments indicated that initiation of iSDR occurs around the origin of normal DNA replication (i.e., *oriC*) (117). This was confirmed by the demonstration that minichromosomes, capable of autonomous replication from their *oriC* site, can undergo replication in the absence of protein synthesis (153). This effect is specific to SOS-induced cells (153). Subsequently, one major origin of iSDR was localized within *oriC* and designated *oriM1* (5). Although *oriM1* overlaps with *oriC*, the intact active *oriC* is not necessary, because mutations that inactivate the OriC activity do not block initiation of iSDR from *oriM1*. In fact, the *oriC* site contains two tandem sites, both of which are independently active as iSDR origins. These are designated *oriM1A* and *oriM1B* (5) (Fig. 1). Interestingly, the two *oriM1* fragments correspond well to the fragments that were shown to bind with high affinity to outer membrane preparations in vitro (132). This coincidence raises the possibility that outer membrane binding plays a role in the regulation of iSDR initiation. Curiously, up to 10% of amplified RecA protein in SOS-induced cells was found in the membrane fraction (68, 81); RecA protein must be activated to the RecA* form to become associated with the membrane (64). iSDR also requires RecA* (see below). The relationship, if any, of this RecA* membrane binding to iSDR is not known.

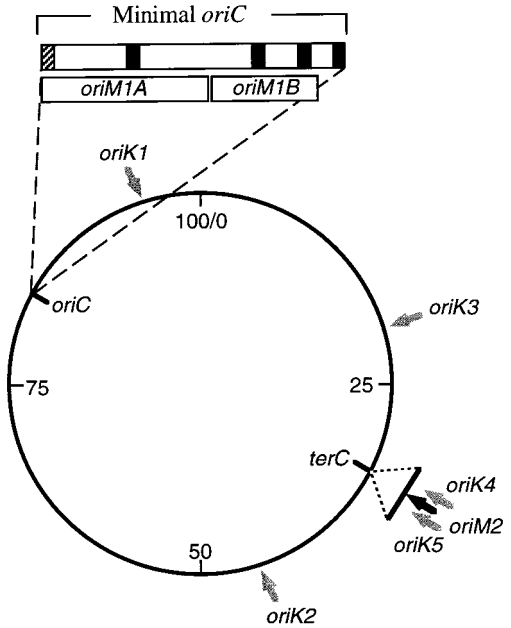

FIG. 1. Map of *oriM* and *oriK* sites. The map locations of *oriC*, *terC*, and *oriM* and *oriK* sites are shown on the *E. coli* chromosome. The *oriM1A* and *oriM1B* sites are indicated within the minimal *oriC*. The hatched and solid rectangles are the AT-rich cluster and DnaA boxes, respectively.

TABLE 2. Gene product requirements for three initiation systems

Gene	Function, activity	Requirement for[a]:		
		oriC	iSDR	cSDR
dnaA	*oriC* binding and priming	+	− (32, 153)	− (124)
dnaB	Helicase	+	+ (104, 118)	+ (112)
dnaC	Complex with DnaB	+	+ (118, 158)	+ (105)
dnaG	Primase	+	+ (118)	+ (112)
dnaE	DNA Pol III core (α subunit)	+	+ (118)	+ (27)
dnaQ	DNA Pol III core (ε subunit)	+	?	?
holE	DNA Pol III core (θ subunit)	+	?	?
dnaX	τ subunit, dimerizes core	+	?	?
dnaX	γ subunit	+	?	?
holA	δ subunit	+	?	?
holB	δ′ subunit	+	?	?
holC	χ subunit	+	?	?
holD	ψ subunit	+	?	?
	γ complex, loads β clamp			
dnaN	Processivity (β sliding clamp)	+	?	?
dnaT	Priming	−	+ (158)	?
priA	Priming	−	+ (160)	+ (160)
priB	Priming	−	?	?
priC	Priming	−	?	?
polA	DNA Pol I	−	− (119, 202)	+ (119)
polB	DNA Pol II	−	− (119)	− (119)
recA	Pairing/strand exchange	−	+ (8, 138)	+ (120, 238)
recB	RecB subunit of exonuclease V	−	+ (154)	− (79)
recC	RecC subunit of exonuclease V	−	+ (154)	− (112)
recD	RecD subunit of exonuclease V	−	− (7, 8)	− (113)
recF	Gapped DNA binding, ATPase	−	+ (8)	− (113)
recG	Helicase	−	± (7, 8)	− (76)
recJ	Exonuclease	−	± (7, 8)	− (113)
recN	Unknown	−	+ (8)	?
ruvA	Complex with RuvB (helicase)	−	± (7, 8)	− (113)
ruvB	Complex with RuvA (helicase)	−	± (7, 8)	?
ruvC	Resolvase	−	± (7, 8)	− (113)

[a] +, required; −, not required; ±, see the text; ?, not known. For the *oriC* system, see references 98 and 127. References for iSDR and cSDR are given in parentheses.

One major origin activity for iSDR (*oriM1*) is localized to the *oriC* region as mentioned above. It was surprising, therefore, that a strain with the *oriC* region deleted exhibited strong iSDR activity (153). Subsequent marker frequency analysis revealed that another origin is located in *terC*, the region of chromosome replication termination. The site was designated *oriM2* (5) (Fig. 1). Replication from the terminus after thymine starvation was suggested by the premature replication of the terminus region that was detected in earlier density shift experiment (117). Two cryptic lambdoid phages, *rac* and *kim*, are located in the *terC* region of some *E. coli* strains. A functional origin of replication has been cloned from the *rac* prophage (40). It was possible that one or both prophages contain an origin of replication that may be activated by SOS induction; however, deletion of either prophage did not affect the iSDR activity that originates from the *terC* region (153). It is not likely, therefore, that *oriM2* represents a prophage origin of replication.

Although *oriM1* and *oriM2* are most conspicuous, the marker frequency experiments suggested at least two other minor sites for iSDR origins. It is not unlikely that additional sites not detectable by marker frequency analysis are also activated in SOS-induced cells.

Gene Product Requirement

The gene products known to be required for iSDR are listed in Table 2. The list for iSDR is characterized by the absence of requirement for DnaA protein and by the requirement for homologous recombination proteins, which are not essential for initiation at *oriC*.

DnaA independence. DnaA protein is the key protein which binds to the 9-bp repeats (DnaA box) clustered within the minimal *oriC* (Fig. 1) and activates *oriC* for initiation of chromosome replication (reviewed in reference 218). A number of temperature-sensitive *dnaA* mutants which are capable of *oriC* initiation at 30 but not 42°C have been isolated. Ciesla and Jonczyk (32) first demonstrated that iSDR can be induced by UV irradiation in *dnaA46*(Ts) mutants at the restrictive temperature. Additional *dnaA*(Ts) mutations such as *dnaA508*, *dnaA167*, *dnaA5*, *dnaA203*, *dnaA204*, *dnaA205*, and *dnaA211* (140, 153) were also shown to not inhibit iSDR at 42°C. It is known that most *dnaA*(Ts) mutations are leaky. The residual activity at the restrictive temperature is sufficient for the replication of some plasmids (71, 102, 116, 176). A *dnaA*::Tn*10* mutation, which completely inactivates DnaA protein, was demonstrated to not block iSDR (153). This ruled out the possibility that iSDR occurred owing to the residual activity in those *dnaA*(Ts) mutants at the restrictive temperature.

RecA protein. RecA protein promotes self-cleavage of LexA repressor (the coprotease activity) in the process of SOS induction (143, 144; reviewed in reference 250). The protein also catalyzes homologous pairing and strand exchange between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) molecules (the recombinase activity) in the homologous recombination process (166, 212; reviewed in references 127 and 145). Active RecA protein is required not only for induction of iSDR but also for repeated initiation once the activity is induced (138). The function of RecA protein that is required for repeated initiation is the recombinase activity, because SOS-inducing treatments of a *recA* mutant that is defective in recombination but proficient in SOS induction fail to elicit iSDR (8). Genetic studies have indicated that the LexA regulon gene that must be derepressed by SOS induction is *recA* and that RecA protein must be activated to the RecA* form (154, 257). It is not absolutely certain what RecA* represents, but it is most probably a form of RecA protein bound to ssDNA (41, 207). Possible roles of RecA* in iSDR are discussed below (see the section on Possible Roles of iSDR).

RecBCD enzyme. The RecBCD protein is a multifunctional enzyme that specifically recognizes a blunt or near-blunt end of duplex DNA. The activities include sequence-specific recognition of Chi, ATP-dependent nucleolytic degradation of ssDNA and dsDNA, and DNA helicase activity (reviewed in references 127 and 219). Chi is a *cis*-acting octamer sequence that, when encountered by RecBCD, causes attenuation of the nuclease activity of RecBCD but not of the helicase activity (42, 43). Chi was shown to prevent the degradation of linear duplex DNA by the RecBCD nuclease in vivo (36, 135). The helicase activity of RecBCD is thought to be essential for initiation of homologous recombination by generating ssDNA tails that can be assimilated into a homologous DNA duplex by the action of RecA protein. Therefore, Chi stimulates RecBCD-mediated homologous recombination (44, 177, 221; reviewed in references 127 and 178).

recB or *recC* mutants, which are devoid of all activity, are deficient in homologous recombination. On the other hand, in *recD* mutants, homologous recombination is not simply active but stimulated. This is because the *recD* mutation does not affect the helicase activity but enhances the generation of invasive ssDNA tails by inactivating the nonspecific nuclease activity of RecBCD (2, 31, 196, 234). The effects of *recBCD* mutations on iSDR completely mimic those on homologous recombination. Thus, *recB* and *recC* mutations block manifes-

tation of iSDR after SOS induction (154) whereas *recD* mutations stimulate iSDR induction (8). It is most likely that the role of RecBCD in iSDR is to yield ssDNA for RecA-catalyzed D-loop formation.

The nuclease activity of RecBCD can be inactivated after SOS induction without significantly inhibiting the helicase activity. There seem to be two mechanisms that contribute to inactivation of the RecBCD nuclease. First, SOS induction causes partial inhibition of Chi activation without affecting overall recombination levels (197). An SOS-inducible protein may modify the RecBCD enzyme by a direct interaction, converting it to a recombinase that retains the helicase activity but has lost the nuclease activity. The induction of the inhibitor of Chi activation is under RecA-LexA control (197). The second mechanism is the phenomenon that is known as restriction alleviation (37, 187). SOS induction produces an inhibitor of degradation of unmodified duplex DNA by the RecBCD nuclease and other restriction enzymes. The inhibitor attenuates the nuclease activity of RecBCD and blocks the expression of *recB*, *recC*, and *recD* (93). Induction of the inhibitor is regulated by *recA* but not *lexA* (93, 236). Thus, restriction alleviation is induced after UV irradiation in *lexA3*(Ind$^-$) mutants but not in *recA* mutants.

Other *rec* gene products. RecJ protein is a 5′-specific ssDNA exonuclease (149). Like *recD* mutations, *recJ* mutations stimulate iSDR, indicating that RecJ is inhibitory (8). On the other hand, *recF* and *recN* mutations mildly inhibit iSDR, suggesting that these proteins are partially required for iSDR. The RuvAB and RecG proteins are helicases that catalyze branch migration, and the RuvC protein is a nuclease that resolves Holliday junctions in the late stages of homologous recombination (214, 252). The effects of *ruvAB*, *ruvC*, and *recG* mutations on iSDR are twofold. First, these mutations, which block the processing of D-loops, greatly stimulate iSDR initiation (for an explanation, see the section on Evidence for the D-Loop Model, below) (8). Second, they severely inhibit the elongation stage of iSDR because Holliday junctions, left on the chromosome due to abortive recombination, arrest replication fork movement (7).

Priming proteins. The events that must follow strand separation for initiation of duplex DNA replication are (i) loading of the replication fork helicase, DnaB, onto ssDNA and (ii) synthesis of primer RNA by DnaG primase. DnaB and DnaG are essential for iSDR (118), indicating that iSDR utilizes the helicase and primase at the replication fork, as the normal replication fork does. *E. coli* possesses several different pathways for the priming step (reviewed in reference 159). At *oriC*, DnaA protein delivers the DnaB helicase from a DnaB-DnaC complex onto ssDNA (209). Since DnaA is completely dispensable for iSDR initiation, this is clearly not the choice of priming for iSDR. iSDR utilizes a priming system that was initially discovered in the course of in vitro studies of ϕX174 DNA replication (3). This ϕX174-type priming reaction involves several proteins including PriA, PriB, PriC, DnaB, DnaC, DnaT, and DnaG. PriA first recognizes and binds a hairpin structure called *pas* (primosome assembly site) in the melted region of the duplex. The binding activates the ATPase activity of PriA. PriB then binds to the PriA-DNA complex. DnaT, perhaps in conjunction with PriC, loads a DnaB helicase to the PriA-PriB-DNA complex from a DnaB-DnaC complex. DnaG primase subsequently interacts with DnaB, completing the assembly of a primosome.

The first hint of involvement of a ϕX174-type priming in iSDR came when *dnaT*, mutations of which block iSDR (136), was identified as the gene encoding the priming protein, i (161). This was followed by the demonstration that DnaC is also required in iSDR initiation (158). More recently, *priA*::*kan* null mutations were shown to completely inhibit the induction of iSDR (160). PriA protein has, in addition to the primosome assembly function, an ATPase and a helicase activity. A mutant PriA(K230R) that is deficient in the ATPase and helicase activities but is capable of catalyzing primosome assembly in vitro was engineered (266). A plasmid expressing this mutant PriA protein complements the defect of the *priA*::*kan* null mutation in iSDR initiation (114). It is most likely, therefore, that the primosome assembly function of PriA is essential for iSDR. The requirement for PriB and PriC has not been examined. Thus, most, if not all, of the ϕX174-type primosome assembly proteins are involved in the priming of iSDR.

The behavior of the mutant PriA(K230R) described above clearly indicates that the ATPase and helicase activities associated with PriA are nonessential for primosome assembly for iSDR. What, then, is the role(s) played by these activities? Recently, Al-Deib et al. (1) examined suppressor mutations that suppress the sensitivity of a *recG* mutant to DNA damage. A majority of the mutations (*srgA*) mapped in the region of the helicase motifs within *priA*. Therefore, *srgA* mutations specifically inactivate the helicase activity of PriA without affecting the primosome assembly function. Thus, these PriA mutant proteins are like PriA(K230R). It was proposed that RecG helicase promotes the conversion of a D-loop into a Holliday junction by its branch migration activity and that the PriA helicase activity opposes this reaction. The balance between RecG and PriA helicase activities is suggested to be critical. In the absence of RecG helicase, PriA dominates and reduces the efficiency of homologous recombination and DNA repair. The inactivation of the helicase activity of PriA by *srgA* mutations compensates for the absence of RecG, restoring efficient recombination and DNA repair (1).

The requirement for a *pas* (primosome assembly site) in SDR priming is not clear. One of the origins of iSDR is located at the *oriC* region (see above). However, no *pas* site is present within or near this origin. In fact, attempts to identify *pas* sites in the *E. coli* chromosome have thus far been unsuccessful. It was suggested that PriA might interact with ssDNA containing no canonical *pas* sequence (6). PriA protein may recognize some feature(s) of a D-loop for binding. This view is supported by two observations. First, the ATPase activity of PriA, which is activated by binding to *pas*, is dispensable for iSDR priming (see above). Second, pBR322 plasmid replication absolutely requires PriA protein, but the deletion of the *pas* near the origin of replication has only a minor effect (157, 160, 240).

DNA polymerases. The dependence of iSDR on DNA polymerase III (Pol III) is indicated by the requirement for *dnaE*$^+$, although the genes encoding other subunits of Pol III holoenzyme have not been examined (Table 1). DNA Pol II, which is SOS inducible (21, 85), is not essential for iSDR. DNA Pol I, which plays a crucial role in cSDR initiation (see the section on the R-loop model below), is not required for iSDR (119).

Proposed Models for iSDR Initiation

Stable-complex model. The stable-complex model, originally proposed when SDR was discovered (117, 118) and later modified (138, 140), assumes that the DNA replication complex (replisome) assembled at the origin of replication is programmed to self-destruct at the end of each round of replication. Protein synthesis is postulated to be necessary to replenish the unstable factor(s) for replication complex reassembly. The self-destruction is ensured by the presence of a factor (the destructor) in the complex. Under SOS-inducing conditions, a complex can be assembled without this factor and thereby

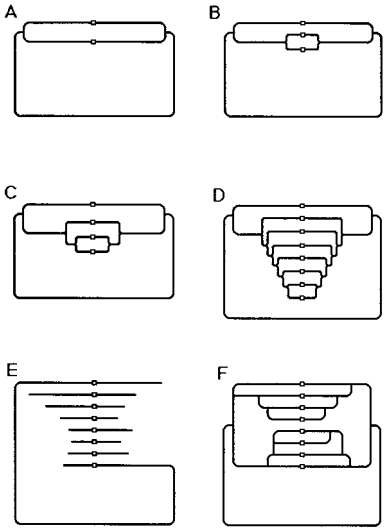

FIG. 2. Mechanism for the accumulation of replication potential for iSDR. The bacterial chromosome is shown as a rectangle with rounded corners. Small open squares designate the replication origins. Explanations are given in the text. Reproduced from reference 133 with permission.

stabilized and reused for the ensuing rounds of replication, giving rise to SDR. Subsequently, two types of mutants, whose SDR behavior was differently affected, were isolated. One type, *sdrT*, constitutively expresses SDR without the use of inducing treatments. It was proposed that *sdrT* could encode a protein that is modified by RecA*. The modified protein, SdrT*, would in turn interact with the destructor to inactivate it, leading to the formation of a stable complex. The *sdrT* mutation was hypothesized to constitutively activate SdrT protein so that the RecA*-mediated modification was dispensable. The second type of mutation, *dnaT*, fails to manifest SDR after the application of inducing treatments (136). The *dnaT* mutation maps very close to *sdrT* but not in the same gene. Therefore, *dnaT* could be a candidate for the gene encoding the destruction factor, and the *dnaT* mutation might render the factor resistant to inactivation by SdrT*, thus leading to obligatory formation of an unstable complex (140).

Although no single line of evidence either proves or disproves this model outright, subsequent studies made it extremely improbable that iSDR arises from such a mechanism. For example, implicit in the model is initiation from the normal origin of replication, *oriC*. iSDR can, in fact, be initiated not only from an *oriC* site that is inactive for normal replication but also from the origin located in the *terC* region (5). Furthermore, *dnaT* has been identified as the gene encoding a priming protein, i (161). Finally, artificially induced double-strand breaks can trigger iSDR (4) (see the section on Evidence for the D-Loop Model, below).

Onion skin model. Conditions that block the movement of replication forks, such as thymine starvation and UV irradiation, cause accumulation of the initiation potential, including DnaA protein, which can be used to multiply initiate at the *oriC* (see, e.g., references 190 and 259). Kuzminov (133) proposed that the multiple initiations under the conditions where the fork progress is severely restricted result in a chromosome that has replication forks bunched up near *oriC* (Fig. 2B to D). Blockade of fork movement would cause replication fork instability, and some of the forks would collapse (Fig. 2E). The RecBCD- and RecA-dependent repair of the collapsed replication forks (see the section on Repair of a Collapsed Replication Fork, below) "is needed to generate an onion-skin structure with the amplified region of the replication origin" (Fig. 2F) (133). This interesting model is clearly inconsistent with several of the well-documented properties of iSDR (summarized in the section on Mode of Replication, above). First, a period of DNA synthesis inhibition, which leads to a 16-fold increase in DNA during subsequent iSDR (117), elevates the copy number of the *oriC* region no more than twofold 15 min after the release of replication block (153). Second, the model predicts both an amplification of the *oriC* region sequences and the preferential use of newly synthesized DNA for the template. During iSDR, no particular sequence is amplified, and the template is chosen at random from the accumulating chromosome pool (117). Third, iSDR can be activated under conditions which do not involve the destabilization of replication forks by replication inhibition, an essential element of the model. The amplification and activation of RecA protein by genetic means without DNA synthesis inhibition is sufficient to activate a significant degree of iSDR (165, 257). Finally, DnaA protein which might be used for the multiple initiations described in the model is completely dispensable for iSDR (153).

D-loop model. iSDR is completely independent from the normally required initiation factors and events such as DnaA protein (32, 140, 153), the *oriC* site (5), concomitant protein synthesis (117), and RNA synthesis (137). This indicates that initiation of iSDR is drastically different from the normal mechanism which initiates replication at *oriC*. The requirement for both the recombinase activity of RecA protein and the helicase activity of RecBCD is most consistent with the idea that iSDR initiation involves a D-loop that is formed by RecA-mediated assimilation of an ssDNA tail which is generated by the action of RecBCD. Because RecBCD requires a duplex DNA end for activity, the process must be triggered by the creation of a DSB. The D-loop model (8) envisions that during SOS induction, a small amount of *oriM*-specific endonuclease is activated and cleaves *oriM* to yield a DSB (Fig. 3). One of the dsDNA ends is unwound by the helicase activity of RecBCD to yield an invasive ssDNA tail that is in turn assimilated by RecA protein to form a D-loop. Under SOS induction, the nuclease activity of RecBCD is inhibited (see the section on RecBCD Enzyme, above); therefore, nucleolytic degradation of DNA strands is not expected. The PriA-mediated priming system loads the DnaB helicase onto the displaced ssDNA. DnaG primase, which interacts with the loaded DnaB, synthesizes an RNA primer for lagging-strand DNA synthesis. The invading ssDNA with a 3′ end may be used to initiate leading-strand synthesis. The replication fork assembled in this manner proceeds to replicate the chromosome unidirectionally. The Holliday junction created as a result of D-loop formation needs to be resolved by the RuvC resolvase. Meanwhile, the ssDNA tail generated from the other end of the DSB also invades one of the two copies of the *oriM* site. The second replication fork carries out unidirectional replication in the direction opposite the first replication fork. Thus, the overall replication is bidirectional. Bidirectional replication proceeds to completion and terminates at *terC*. The product of this process is expected to be a concatemer that consists of two circular chromosomes linked by a linear chromosome (Fig. 3). The concatemer can be resolved by site-specific recombinases (19, 20, 131) into three complete chromosomes (Fig. 3).

Evidence for the D-Loop Model

Central to the D-loop model is the generation of a DSB at the *oriM*. The model predicts that an artificially generated DSB

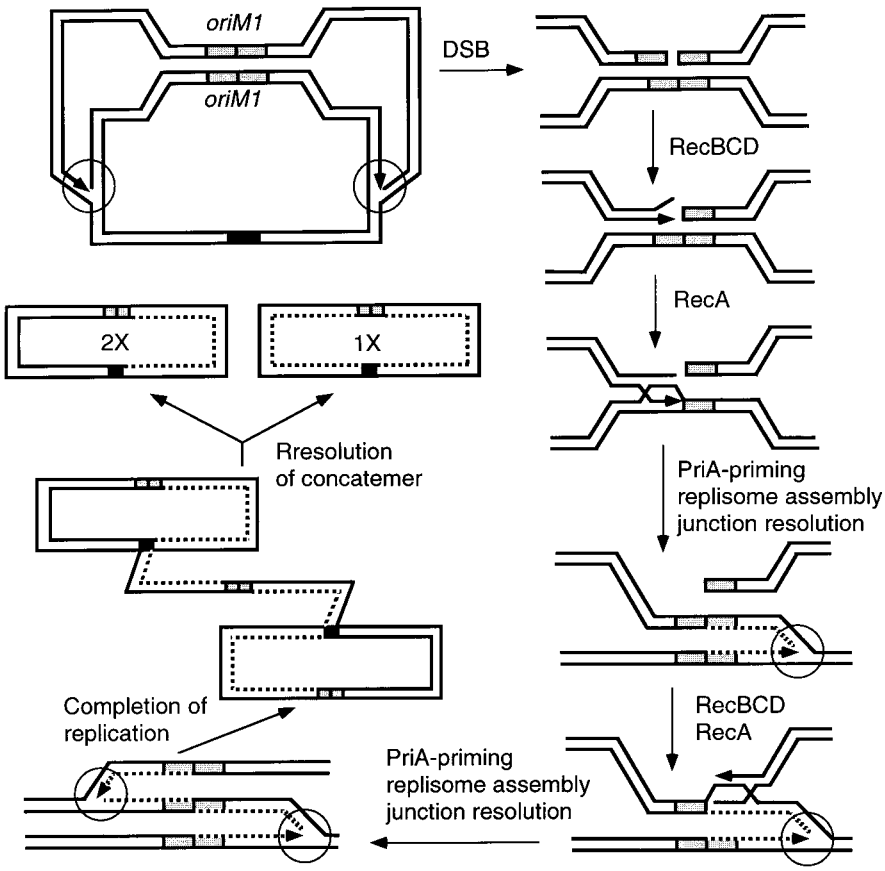

FIG. 3. D-loop model of iSDR initiation. A partially replicated chromosome has two copies of *oriM1* (shaded boxes), one of which is cleaved to yield a DSB. The solid box indicates *terC*, the termination site of normal replication. Newly synthesized DNA strands and replication forks are shown by broken lines and circles, respectively. See the text for details.

triggers chloramphenicol- and rifampin-resistant replication, which should occur without the *oriM* site. This prediction was directly tested by placing λ *cos* site on a plasmid and generating a DSB at the site with λ terminase, which introduces two staggered nicks, 12 bp apart (reviewed in reference 53). Replication of the plasmid in the presence of chloramphenicol and rifampin, which inhibits plasmid replication from the normal origin of replication, was monitored by determining the copy number of the plasmid in SOS-induced cells after controlled synthesis of λ terminase. A limited amount of the terminase synthesized was expected to cleave part of the plasmid population, and the ends generated were expected to trigger replication on intact plasmid molecules. DSBs artificially generated in such a manner indeed triggered origin-independent plasmid replication (4). The major products of the replication were covalently closed circular monomers. This suggests that the replication proceeds by the θ mode. The replication depended both on the presence of a *cos* site in the plasmid and on synthesis of the terminase.

Thus, artificial DSBs trigger an SDR-like replication of a plasmid that lacks *oriM* sites, providing strong support for the model. In addition, the following observations support the model. First, the D-loop that acts as an origin for iSDR is most probably structurally identical to the D-loop that is formed as an early intermediate in homologous recombination (166, 212). Thus, the iSDR and homologous recombination processes are expected to compete for D-loops. In homologous recombination, D-loops are further processed by RuvAB, RecG, and RuvC proteins to yield recombinant molecules. As expected from the model, iSDR activity was found to be significantly stimulated by *ruvA*, *ruvB*, *ruvC*, and *recG* mutations, which block the processing of the intermediate (7). Second, the model predicts that the presence of extra copies of *oriM* would enhance the iSDR initiation frequency because the extra copies could provide additional ends that could trigger replication. This prediction was verified by the demonstration that introduction of a plasmid carrying the *oriM1* site stimulates the iSDR activity (5). The stimulation is origin specific in that the stimulatory effect of the extra copies of *oriM1* cannot be seen in the strain that has the *oriM1* site deleted. However, the level of stimulation is not proportional to the copy number of *oriM1* introduced (5). This suggests that some *trans*-acting factor (e.g., the hypothetical endonuclease activity) is limiting in the initiation reaction. Third, after a period of thymine starvation, a drastic change in the chromosomal DNA structure is induced such that DNA migrates extremely slowly or does not migrate at all through agarose upon electrophoresis (179). The nonmigrating DNA is enriched with structures containing ssDNA gaps or tails and with highly branched structures. Such structures are largely associated with DNA fragments that contain *oriC*, and their formation depends highly on *recA*$^+$ (179). It is likely that at least part of such DNA results from chromosome replication initiated at D-loops formed at *oriM1*. Fourth, as described previously, iSDR depends very strongly on PriA-catalyzed priming (114, 160).

Roles of SOS in iSDR Initiation

In the D-loop model, the role of SOS induction is twofold. One role is to activate the hypothetical endonuclease specific to *oriM*. Previously, thymine starvation was reported to generate DSBs in the chromosome (265) and cause activation of an endonuclease activity (62). The specificity, if any, of the endonuclease for certain sequences including *oriM* has not been examined. The second role of SOS induction is inhibition of the nuclease activity of RecBCD (93, 187, 197), which results in the stabilization of invasive ssDNA tails and the consequential enhancement of iSDR initiation. This role of SOS induction was deduced from the following observations. In the model replication system where origin-independent plasmid replication is triggered by artificial DSBs (see above), SOS induction was still necessary for the replication. The SOS induction was rendered dispensable by a *recD* mutation, which inactivates the RecBCD nuclease activity, or by inclusion in the plasmid of a Chi site which attenuates the nuclease (4). Furthermore, the origin-independent replication was detected without SOS induction in a *recBC sbcA* mutant which is devoid of the nuclease activity of RecBCD but is proficient for homologous recombination owing to the activation of another recombination pathway, the RecE pathway (4).

RECOMBINATION-DEPENDENT DNA REPLICATION

RDR Model

A crucial observation made in studies with the plasmid model system (see the section on Evidence for the D-Loop Model, above) is that an SDR-like activity can be activated without SOS induction when certain specific conditions are met. These conditions are (i) generation of a duplex DNA end and (ii) attenuation of the RecBCD nuclease activity. When encountered by RecBCD, Chi attenuates the nuclease activity of the RecBCD enzyme (42). When the plasmid contains a Chi site, origin-independent plasmid replication can be triggered by an artificial DSB even in normal cells not induced for SOS. This replication absolutely depends on $recA^+$, $recB^+$, and $recC^+$ (4). The observation led to the proposal that RecA- and RecBCD-dependent replication could routinely occur in normal cells when a duplex DNA end is generated and the linearized duplex DNA is protected from the RecBCD nuclease by the presence of Chi in the duplex. The homologous recombination function-dependent replication triggered by a duplex DNA end is designated recombination-dependent DNA replication (RDR) (4).

iSDR Is a Special Type of RDR

From the discussions above, it is easy to envision that iSDR and RDR are mechanistically identical. However, there are two major differences. First, in iSDR, attenuation of RecBCD nuclease is achieved specifically by the activation of inhibitors of the RecBCD nuclease as part of the SOS response (93, 197). The second difference is the site where replication is initiated. Clearly, iSDR originates mainly from specific origins (*oriM*). On the other hand, RDR can be initiated at a site where a D-loop can be formed. In wild-type cells, the site can be at or near a Chi site where degradation of duplex DNA is stopped and an invasive ssDNA tail is formed (42). In *recBC sbcB sbcC* mutants, RecQ helicase, together with RecJ nuclease, may produce invasive ssDNA independent of Chi (127). In *recBC sbcA* cells, exonuclease VIII, activated by the *sbcA* mutation, may generate an ssDNA tail irrespective of Chi (4). Thus, iSDR is a special type of RDR.

POSSIBLE ROLES OF iSDR

Damage-Resistant Replication

E. coli polymerases are extremely sensitive to pyrimidine dimers (243). DNA replication in cells induced for iSDR is considerably more resistant to UV irradiation than is the replication in the presence of chloramphenicol in uninduced cells. Thus, at a UV dose that completely inhibits normal replication in the absence of protein synthesis, the rate of iSDR, after a short lag period, recovers to the rate that is found in unirradiated controls (122). About 80% of the DNA synthesis after UV irradiation occurs in the semiconservative manner under these conditions. A similar recovery of DNA synthesis can be seen in a *uvrA6* mutant which is excision repair deficient. Thus, iSDR can tolerate more pyrimidine dimers in templates than can normal replication (122, 208). The mechanism of the tolerance is not understood. Since a significant fraction of UV-induced damage (e.g., pyrimidine dimers) is converted to DSBs (22, 251) and since iSDR is a type of RDR which is initiated by DSBs as described above, it is likely that at least part of the dimer-resistant replication represents RDR.

There is solid evidence that newly synthesized DNA following UV irradiation contains gaps opposite dimers (201). It is possible, therefore, that the replisome of iSDR that is stalled at a dimer is able to restart DNA synthesis downstream of the dimer. It was reported that SOS induction enables stalled replication to restart (50, 99, 258). This induced replisome reactivation (IRR) shares some of the characteristics of iSDR: it is inducible upon SOS response, can occur in the presence of chloramphenicol and rifampin, and requires RecA protein. Furthermore, like in iSDR, *recA* is the only gene controlled by LexA that must be amplified for IRR (258). Hence, some aspects of SDR were incorporated into a proposed model for IRR (29, 99). However, there are some critical differences between the two activities. Whereas iSDR strictly depends on $recB^+$ and $recC^+$ (154), IRR does not require $recB^+$ (99). Unlike iSDR, amplified RecA protein is not sufficient for IRR: one additional gene product (Irr factor) is required. Despite the evidence implicating an RNase HI inhibitor in the recovery process of stalled replisomes, no change in RNase HI activity was detected during or after the SOS response (17, 29). Furthermore, little effect of overproduction of RNase HI on the inducibility of iSDR was seen (17). These considerations make it unlikely that iSDR and IRR are the same activity. It is possible, however, that the two processes share a basic inducible activity.

Error-Prone Replication

iSDR appears to be error prone (138). The proposal is based mainly on three observations. (i) Cells that are induced for iSDR show a high rate of spontaneous mutations. (ii) This high rate of mutation is drastically reduced by a *dnaT* mutation, which blocks induction of iSDR. (iii) Mutagenesis with methyl methanesulfonate, which also induces iSDR, is inhibited by the *dnaT* mutation. In general, the conditions that prevent the induction of iSDR significantly reduce the mutation rate (138). The observations raise the possibility that iSDR is the error-prone replication that is associated with the UmuD′C-dependent mutagenesis in SOS-induced cells (reviewed in reference 250). Consistent with this proposal is the requirement for the activated form of RecA protein, RecA*, which parallels the requirements defined for SOS mutagenesis (51, 61, 181, 228). In addition to the two well-characterized roles of RecA protein in SOS mutagenesis, i.e., cleavage of LexA and conversion of UmuD to UmuD′, RecA* plays a third role in SOS mutagen-

esis. The precise nature of the third role is unknown, but the activated RecA may act directly, perhaps by interacting with and modifying a DNA replication complex. The modification may allow efficient replication of damaged DNA at the cost of increased infidelity (49). It is possible that this modification endows iSDR with the characteristics of UV resistance (see above) and error-prone DNA replication. However, iSDR can be induced in *umuC* mutants, which no longer exhibit mutagenesis (unpublished data quoted in reference 249). It could mean that iSDR is necessary but insufficient for the mutagenesis. Alternatively, the replisome assembled at a D-loop is inherently error prone (e.g., inhibition of the editing exonuclease) or a mismatch repair system associated with the replication is compromised under the condition. In this context, it is noteworthy that DSB repair has been reported to cause mutations in nearby sequences in yeast and mammalian cells (48, 223).

Possible Role in Adaptive Mutation

Certain mutations can occur in nongrowing *E. coli* cells, and these mutations appear to be adaptive because the only mutations recovered are those that permit the cells to grow (reviewed in references 56 and 199). Analyses of mutations recovered led to the conclusion that mutations arise as a result of replication error in a state in which methyl-directed mismatch repair is suppressed (59, 148, 200). DNA Pol III is responsible for most of the errors, and a Δ*polB* mutation (inactivating DNA Pol II) stimulates adaptive mutation (57). Adaptive mutation depends on $recA^+$ (26). It is severely inactivated by the *recB* and *recC* mutations but is stimulated by the *recD* mutation (72). The shared characteristic in the requirement for RecA protein and RecBCD enzyme raises the possibility that iSDR is involved in adaptive mutation (159). iSDR is a form of replication that can occur in nongrowing cells and appears to be mutagenic (see above). iSDR employs DNA Pol III but not DNA Pol II (119). These characteristics of iSDR are consistent with the idea. Recently, several models have been proposed for the mechanism of adaptive mutation (60, 73, 134). These models propose both generation of a D-loop by the actions of RecA protein and RecBCD enzyme and initiation of semiconservative replication at the D-loop. These schemes are very similar to the D-loop model of iSDR described above. It is likely that recombination-dependent replication plays a crucial role in the emergence of adaptive mutation. The predicted requirement for PriA in adaptive mutation has not been tested.

RDR AND DOUBLE-STRAND BREAK REPAIR

Role of RDR in DSB Repair

In the plasmid model system for RDR described above, it is envisioned that a donor plasmid that is cleaved by the terminase triggers a semiconservative θ mode of replication on another molecule (4). Completion of the replication would yield two circular plasmid molecules. To gain a net increase in plasmid yield, it would seem necessary that the donor plasmid molecule be recovered as an intact circle. This implies that the DSB in the donor plasmid might be repaired by the process. Thus, RDR probably leads to DSB repair.

RDR-mediated DSB repair model. When a chromosome suffers a DSB (Fig. 4a), RecBCD recognizes the ends and begins degrading the duplex, converting the DSB into a double-strand gap (route A). Chi sites are overrepresented in the *E. coli* chromosome (about one every 4 kb on the average) (52), and thus RecBCD is expected to encounter a Chi site

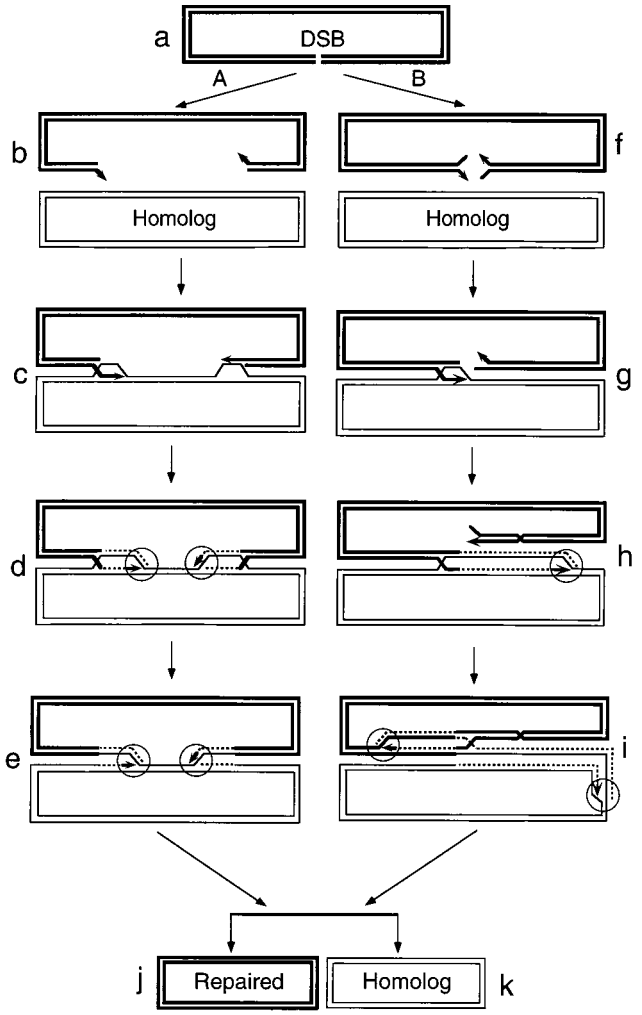

FIG. 4. RDR-mediated DSB repair. DNA strands of a chromosome that has just suffered DSB and a homolog chromosome are indicated by thick and thin lines, respectively. Newly synthesized DNA strands and replication forks are shown by broken lines and circles, respectively. See the text for details.

before it degrades the duplex too far. When encountering a Chi site, the nuclease activity of RecBCD is attenuated, producing an invasive ssDNA tail at each end (Fig. 4b). The tails are assimilated into a homolog by RecA protein to form a D-loop at each end (Fig. 4c). The replication forks assembled at the D-loops after the PriA-mediated priming process replicate toward each other (Fig. 4d). Meanwhile, RuvC resolvase resolves the two Holliday junctions (Fig. 4e). When the two forks meet and complete the replication, the process yields a repaired chromosome and a homolog (Fig. 4j and k). In this mode of repair, the extent of semiconservative DNA replication is limited to filling the gap generated by RecBCD processing.

Efficient DSB repair requires the induction of SOS (see below). As discussed above (see the section on Gene Product Requirement, RecBCD Enzyme), Chi activation is partially inhibited in SOS-induced cells (197) and thus the attenuation of RecBCD nuclease activity by Chi may not occur effectively. Instead, inhibitors of the RecBCD nuclease that convert the enzyme into a helicase with no DNA-degrading activity are induced (93, 197). Because of this, conversion of a DSB into a double-strand gap is not likely. Thus, in SOS-induced cells, a

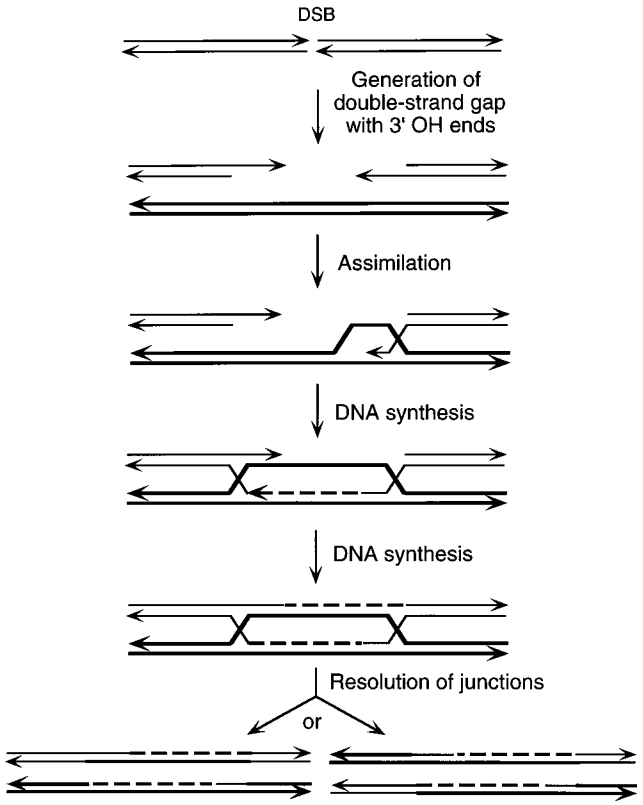

FIG. 5. Conventional model of double-strand gap repair. Duplex DNA with a DSB and homolog duplex DNA are shown by thin and thick lines, respectively. Broken lines indicate newly synthesized DNA. Adapted from reference 229.

more likely sequence of events would be the following (Fig. 4, route B) (110). The altered RecBCD enzyme recognizes the duplex ends and unwinds the duplex to yield recombinogenic ssDNA tails (Fig. 4f). A D-loop is formed at one end, and a replication fork assembled at the site begins replication in one direction (Fig. 4g and h). Subsequently, the second end invades one of the duplicated chromosomes while the first fork continues to replicate the remainder of the chromosome (Fig. 4i). In this case, the involvement of DNA replication is extensive and the product would have a complex structure requiring the resolution of Holliday junctions by RuvC resolvase aided by RuvAB and/or RecG helicase. The resulting concatemer can be eventually resolved into monomers (Fig. 4j and k) in a manner similar to that described for Fig. 3. The possibility that the ends generated by DSBs trigger DNA replication leading to DSB repair has been considered previously (220, 235).

Resnick (193) proposed a model for the repair of radiation-induced DSBs and pointed out that the same process could accomplish homologous recombination. Szostak et al. (229) refined Resnik's model to explain meiotic recombination in lower eukaryotes. A major difference between the two models is that whereas the former postulates 5′→3′ nucleolytic degradation of one strand to expose 3′ ssDNA ends, the latter model involves the generation of a gap flanked by 3′ ssDNA ends (Fig. 5). Thus, it can be said that the former is a DSB repair model while the latter is a double-strand gap repair model (235). The RDR-mediated DSB repair model described above differs from these conventional DSB repair models in at least two very important aspects. First, in the new model, the DSB repair process could involve extensive semiconservative DNA replication. In contrast, the conventional models postulate an involvement of a limited extent of ssDNA repair synthesis to fill the missing DNA (Fig. 5). Second, in SOS-induced *E. coli* cells, no significant extent of RecBCD processing may occur because of inhibition of the nuclease activity of RecBCD. Therefore, in damaged SOS-induced cells, DSB repair is most likely to proceed as outlined in Fig. 4, route B.

Evidence for the RDR-mediated DSB repair model. The *priA* null mutation blocks iSDR (see the section on Gene Product Requirement, Priming Proteins, above). The introduction of plasmid expressing a mutant PriA protein, PriA(K230R), that is capable of primosome assembly despite the lack of the ATPase and helicase activities normally associated with wild-type PriA (266) can rectify this defect of the *priA* null mutant (114). These results strongly suggest that RDR requires PriA-mediated priming, although the effect of the *priA* null mutation on RDR has not been tested directly (160). The *priA* null mutants were shown to be hypersensitive to gamma rays and mitomycin, which cause DSBs in the chromosome (114). The result suggests that *priA* null mutants are deficient in DSB repair. It was shown that the mutant PriA(K230R) can complement the DSB repair defect of *priA* null mutants (114). Similarly, an extragenic suppressor mutation, *spa-47*, of the *priA* null mutation, which partially restores iSDR inducibility to *priA* null mutants, also suppresses the hypersensitivity to mitomycin. The *spa-47* mutation maps at or very close to *dnaC* (114). Since *dnaC* and *dnaT* constitute an operon and both gene products are involved in the primosome assembly (see the section on Gene Product Requirement, Priming Proteins, above), it is likely that the suppressor mutation changes the structure of DnaC or DnaT to allow the assembly of active primosomes in the complete absence of PriA. Taken together, these results indicate that a large part of DSB repair proceeds in a manner that involves RDR as described in the above model.

The RDR-mediated DSB repair model is consistent with previous observations. First, repair of ionizing radiation-induced DSBs absolutely depends on *recA*$^+$, *recB*$^+$, and *recC*$^+$ (128, 206) and the availability of duplicated chromosomes (128). Second, RDR (4) and DSB repair (189, 206) both are dependent on RecN. Third, the analysis of DSB repair of the DNA from X-irradiated cells by neutral sucrose gradient centrifugation revealed that slow-sedimenting DNA resulting from DSBs was converted to fast-sedimenting material as the repair proceeded (205). Two interesting observations were that the fast-sedimenting DNA sedimented much faster than non-irradiated DNA and that it took several hours to return to the normal-sized DNA. It is likely that the fast-sedimenting material represents DNA that is being replicated by RDR after repair of DSBs (see e.g., Fig. 4i).

Kobayashi and coworkers developed an elegant assay system for DSB repair in *E. coli* (103, 264). The pBR322-based construct is designed in such a way that when a DSB (or double-strand gap) is repaired, an intact *neo* gene is generated from two copies of imperfect alleles that each have a small deletion at the opposite ends. Thus, the frequency of DSB repair can be measured by selecting for resistance to kanamycin (conferred by the *neo* gene) upon transformation with the probe. The probe also allows easy analysis of the products to determine if crossing-over has accompanied the gene conversion (i.e., *neo* to *neo*$^+$). However, DSB repair was detectable with this system only in *recBC sbcA* cells, where the RecBCD pathway of homologous recombination is inactive and the RecE pathway is operating (231). The repair of DSB detected with this system was not dependent on the gene products such as RecA and RecN, which are known to be essential for DSB repair (see

above). In fact, DSB repair could not be seen in wild-type cells, where the RecBCD pathway is active (231). This is most probably due to degradation of the probe by RecBCD exonuclease, because the probe did not contain Chi sites for protection. Thus, the repair detected with this system seems to be a subset of DSB repair which occurs under very special conditions. Any relevance to the major DSB repair process as discussed above is doubtful.

Efficient DSB Repair Requires SOS Induction

The repair of ionizing radiation-induced DSBs is an inducible function (24, 129) and is completely inhibited by a *lexA*(Ind$^-$) mutation (206). These observations indicate that although RDR can occur in normal cells (not induced for SOS) (see the section on RDR Model, above), effective DSB repair requires additional factors that are inducible by SOS. In normal cells, the RecBCD nuclease is attenuated by the *cis*-acting Chi sites. The action of a Chi site is orientation dependent: only when approached from the 3′ side of the octamer (5′-GCTGGTGG-3′) is Chi recognized (232) and the nuclease activity of RecBCD attenuated (42). Thus, only properly oriented Chi sites can protect duplex DNA from RecBCD degradation. Close examinations of Chi sites in the chromosome showed that at about 90% of the time, Chi is oriented toward the origin of replication, *oriC*, protecting that portion of the duplex from RecBCD degradation (25, 167). In other words, when a DSB occurs, the *oriC*-proximal chromosome arm can be protected but the *terC*-proximal arm is much less likely to be protected. Even the protection of the *oriC*-proximal arm is not complete, because the nuclease activity of RecBCD is attenuated only 20 to 30% of the time when it encounters a properly oriented Chi site (43, 222, 233, 263). Consequently, repair of DSBs would be very inefficient. The inhibition of the RecBCD nuclease by SOS induction (93, 197), on the other hand, should result in equal protection of both ends and could promote efficient repair as outlined in the RDR-mediated DSB repair model (Fig. 4, route B).

Activation of one of the inhibitors of RecBCD nuclease is not regulated by LexA repressor, and therefore it can be induced in *lexA*(Ind$^-$) mutants (93, 236). Since the DSB repair capacity cannot be induced in *lexA*(Ind$^-$) mutants (206), it implies that efficient DSB repair requires either amplification of some proteins such as RecA and RuvAB, which are under LexA control (127), or activation of a new gene product(s) that is repressed by LexA, or both.

recA polA LETHALITY AND REPLICATION FORK COLLAPSE

Repair of a Collapsed Replication Fork

More than 20 years ago, Skalka (217) proposed that breakage of a chromosome arm could result from replication fork collapse due to a fork running into a nick or gap left in the template (Fig. 6). The asymmetric distribution of Chi sites along the chromosome (25, 167) led to the proposal that Chi is evolutionarily designed to protect the *oriC*-proximal arm when it is broken off at the replication fork due to replication fork collapse (134, 135). A unique feature of RDR-mediated DSB repair is that it not only repairs DSBs but also regenerates a replication fork at the site. Thus, RDR-mediated repair is ideally suited for the repair of a collapsed replication fork (4). The end of the chromosome arm that is broken off could be recognized by RecBCD, and the arm would be degraded up to a nearest Chi, where an ssDNA tail might be created (Fig. 6). Formation of a D-loop by assimilation of the ssDNA into a homolog and PriA-mediated priming followed by replication protein assembly should effectively restore the replication fork (4, 134).

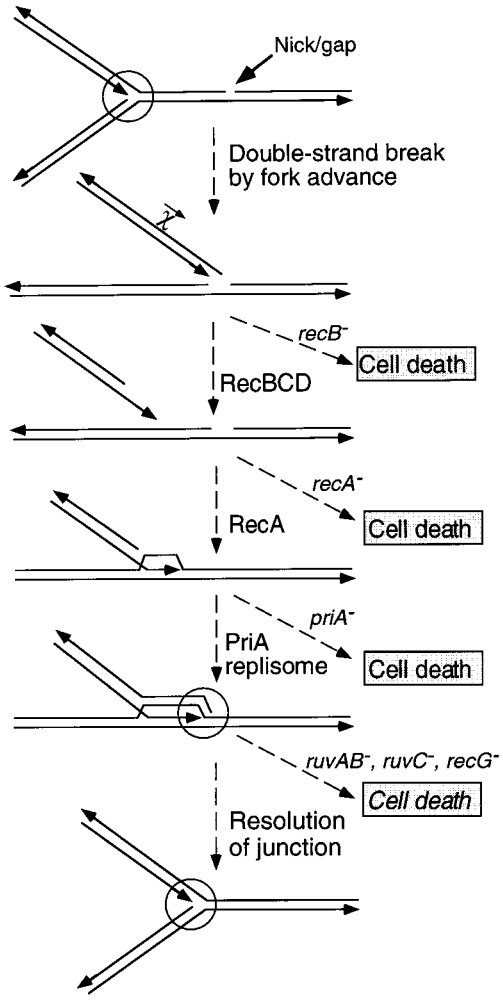

FIG. 6. Mechanisms of *polA recA* and *polA recB* lethality. Thin lines represent DNA strands, and the arrowheads indicate the 3′ end. Circles indicate the locations of replication forks. See the text for details.

Mechanism of *recA polA* Lethality

The *polA* gene encodes DNA Pol I, which plays an important role in the processing of Okazaki fragments in lagging-strand DNA synthesis (126). The 5′ → 3′ exonuclease activity of DNA Pol I effectively removes the RNA primer, and the polymerase activity replaces it with the DNA moiety. The combination of a *polA* and a *recA* mutation is lethal (67, 171). The combination of *polA* and *recB* mutations is also lethal (171). The lethality has attracted the attention of a number of molecular geneticists, partly because it is thought that solution of the problem might reveal the elusive relationship between DNA replication and homologous recombination. Recent studies suggested that the defect of *polA* mutants in Okazaki fragment processing results in the accumulation of nicks and gaps during lagging-strand synthesis (28, 227). When a replication fork encounters

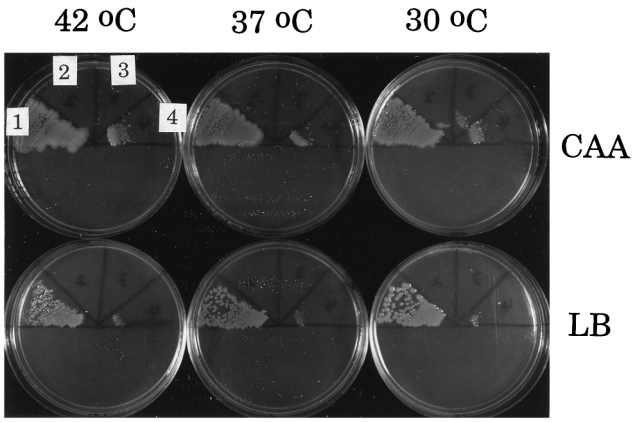

FIG. 7. Temperature-sensitive and broth-sensitive growth of *polA priA* double mutants. About 10^5 cells were spotted on CAA (M9 plus glucose supplemented with Casamino Acids) and LB (Luria broth) plates and spread with toothpicks. The plates were incubated at 30, 37, and 42°C for 52 h. Sectors: 1, *polA12 priA*$^+$; 2, *polA12 priA1*; 3, *polA12 priA2*; *polA*$^+$ *priA1*.

a discontinuity in the template, replication fork collapse would result (Fig. 6). The collapsed fork, which could be efficiently restored by RDR-mediated repair in wild-type cells, is blocked by the *recB* or *recA* mutation, leading to cell death (Fig. 6) (28). The model predicted that the *priA* mutation, which blocks the priming step of RDR, should render *polA* mutants inviable. When the *priA* mutation was combined with *polA12*(Ts), the double mutant was indeed very temperature sensitive: it could not grow even at 37°C (Fig. 7) (141). Growth of the mutant was also extremely sensitive to nutritionally rich media, like that of the parental *priA* single mutant (160), and the mutant failed to grow at 30°C in Luria broth (Fig. 7). The results suggest that repair of the collapsed replication fork requires PriA-catalyzed priming and support the above model.

Completion of the repair would require resolution of the Holliday junction near the reestablished replication fork (Fig. 6). Holliday junctions are resolved by RuvC resolvase, but the resolution in vitro is inefficient (15, 230). It was suggested that efficient resolution of Holliday junctions in vivo requires branch migration, which is catalyzed by RuvAB helicase and RecG helicase (213, 253). Mutations that block these processes in the late stage of homologous recombination are therefore expected to reduce the viability of *polA* mutants. *ruvA*, *ruvB*, *ruvC* (82), and *recG* mutations (76) were indeed shown to cause temperature-sensitive growth when combined with the *polA12*(Ts) mutation. The genetic requirements of the RecA-dependent, SOS-inducible DSB repair pathway are summarized in Fig. 8 (for details on the RecA-independent pathway, see below).

Replication Fork Collapse and Broth Sensitivity

The effects of certain mutations are drastically exacerbated and may even result in cell death when the mutant is grown in nutritionally rich media such as Luria broth. This phenomenon is termed broth sensitivity. Some of the broth sensitivity phenotypes can now be understood in terms of replication fork collapse. Deletion of the entire *polA* gene is lethal in rich medium but not in minimal medium (91). The fact that the Δ*polA* mutant is viable in minimal medium implies that a secondary system can process Okazaki fragments, albeit inefficiently. The distance between a leading replication fork and the following fork depends on how often chromosome replication is initiated at *oriC* during a cell cycle (247). In minimal medium containing glucose as the sole carbon source, a new round of replication is initiated when the leading fork travels about halfway on the chromosome. Thus, before the next fork catches up, the secondary processing system could have sufficient time to minimize the number of nicks and gaps which would cause infrequent DSBs that could still be repaired by RecA-dependent DSB repair. On the other hand, cells growing in rich medium have only a short time for Okazaki fragment processing such that the number of DSBs overwhelms the DSB repair capacity, leading to cell death. A similar explanation can be applied to the broth sensitivity seen with *rnhA polA* mutants (115) and with *priA* mutants (Fig. 7) (160).

RecA-INDEPENDENT REPAIR OF DOUBLE-STRAND BREAKS

Suppression of *recA polA* Lethality by *lexA*(Def)

The *lexA*(Def) mutation completely inactivates the LexA repressor, derepressing the LexA regulon (250). *lexA*(Def) was found to suppress the *recA polA* lethality (28). Thus, *recA200*(Ts) *polA lexA*(Def) mutants are temperature resistant at the restrictive temperature for RecA200. This implies that derepression of one or more LexA regulon genes alleviates the defect in either RecA recombinase or DNA Pol I. This inducible system was designated the Srp (suppression of *recA polA* lethality) pathway. The suppression does not come from an improved Okazaki fragment processing because the joining of Okazaki fragments is not significantly faster in *lexA*(Def) cells than in *lexA*$^+$ cells (28). Rather, the defect in the RecA protein is alleviated. This conclusion is based on the following observations. First, the *lexA*(Def) mutation restores homologous recombination despite the defect in the RecA protein. For example, the P1 transduction frequency is reduced 1,000-fold in Δ*recA* mutants compared to *recA*$^+$, but *lexA*(Def) elevates the level to more than 7% of the wild-type level. Consistent with this, *lexA*(Def) desensitizes Δ*recA* mutants to UV light by 1,000-fold, suggesting a boosting of the recombinational repair capacity in Δ*recA* mutants (28). Second, *lexA*(Def) does not suppress the *recB polA* lethality. The suppression is therefore rather specific to the *recA* defect (92). Thus, the Srp pathway is capable of DSB repair in the complete absence of the strand exchange activity of RecA protein.

Two Pathways for DSB Repair

The recent analysis of the Srp system has suggested that Srp is another pathway of DSB repair that may play a different role from that of RecA-dependent DSB repair in the survival of *E. coli* (92). To identify the gene (*srp*) responsible for the suppression, a temperature-sensitive mutant was isolated from a population of *recA polA lexA*(Def) mutant cells randomly mutagenized with a mini::Tn*10* transposon. This mutation was designated *srp-529*::miniTn*10*. The mutation was cloned, and a short sequence next to the Tn*10* insertion was determined. A

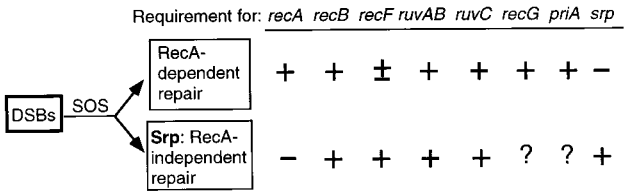

FIG. 8. Two pathways of DSB repair in *E. coli*. The genetic requirements of two inducible DSB repair pathways are compared.

search in the gene banks indicated that the sequence is located near the *pck* gene at around 76 min on the chromosome (16). A plasmid carrying a wild-type sequence complemented the *srp-529*::miniTn*10* mutation for the suppressing activity. This gene was designated *srp*. Promoter activities were localized about 2 kb upstream of the beginning of *srp*. In fact, upstream of *srp* are two more genes; together, the three genes (encoding 27- to 15-kDa Srp proteins) constitutes an operon. At least two promoters were identified. One is inducible about fivefold with mitomycin and UV irradiation, and the other is strongly activated when cells enter the stationary phase. The activation of the second promoter depends largely on σ^s (the *rpoS* gene product). Together, the operon is strongly expressed in the stationary phase in the presence of an SOS inducer (92). Previously, it was demonstrated that when cells growing rapidly in rich medium enter the stationary phase, the SOS response is activated to a significant degree (77). Thus, it is likely that the *srp* operon is strongly activated in stationary-phase cells.

Figure 8 compares the genetic requirements of the RecA-dependent and RecA-independent (Srp) pathways of DSB repair. Besides the difference in the requirement for RecA and Srp, the two pathways also differ in their dependence on RecF. Thus, the Srp pathway depends completely on *recF*$^+$ (28) whereas *recF* mutations affect the RecA-dependent pathway only partially (206). These two pathways of DSB repair may be distinct activities and may play different roles in DSB repair: RecA-dependent DSB repair may be responsible primarily for DSBs generated in actively growing cells, while the Srp pathway may function in stationary-phase cells.

Is Srp a RecA Analog?

RecO protein catalyzes the assimilation of ssDNA into homologous dsDNA and partially complements the UV repair deficiency of Δ*recA* mutants when overexpressed (150). Thus, RecO might be a RecA analog. RecT protein promotes the renaturation of complementary ssDNA (69). RecE and RecT can catalyze a certain type of homologous recombination (i.e., circular plasmid recombination) in a RecA-independent manner (54). In conjunction with other proteins, therefore, RecT might function in place of RecA protein. As mentioned above, the derepression of the *srp* operon by *lexA*(Def) restores P1 transduction proficiency to Δ*recA* mutants to a remarkable extent. Southern blot hybridization analysis of the chromosome of the transductants at the site of transduction revealed that the recipient allele was replaced by the donor allele, indicating that the transductants were the products of homologous recombination rather than those of insertional events (28). Thus, the derepression of Srp allows homologous recombination to occur in the complete absence of RecA protein. It is possible, therefore, that the *srp* operon encodes a functional analog of RecA protein.

REPLICATIVE HOMOLOGOUS RECOMBINATION

One other important implication of the discovery of RDR in *E. coli* is that it raises the possibility of recombinant formation involving extensive DNA replication (Fig. 9, route A). The model, termed replicative homologous recombination, has been described in detail elsewhere (109, 114, 204). Both ends of a fragment of donor dsDNA brought into a recipient cell during P1 transduction or conjugation engage themselves in the formation of D-loops, one at each end (Fig. 9a), and a replication fork can be assembled at each site (Fig. 9b). The two replication forks so formed continue to replicate, copying the remainder of the entire chromosome in the opposite directions. The consequence of such a process after resolution of Holliday junctions (Fig. 9c) and completion of replication is that the donor DNA fragment is integrated into a newly replicated chromosome (Fig. 9d). This is in sharp contrast to conventional models of homologous recombination (see, e.g., reference 219), which postulate no or very limited involvement of DNA synthesis in the process (Fig. 9, route B). Interestingly, early density shift experiments to analyze the fate, in the recipient cell, of transferred DNA fragments during Hfr mating provided evidence for covalent joining of donor DNA to newly synthesized strands (216), which is predicted by the replicative homologous recombination model.

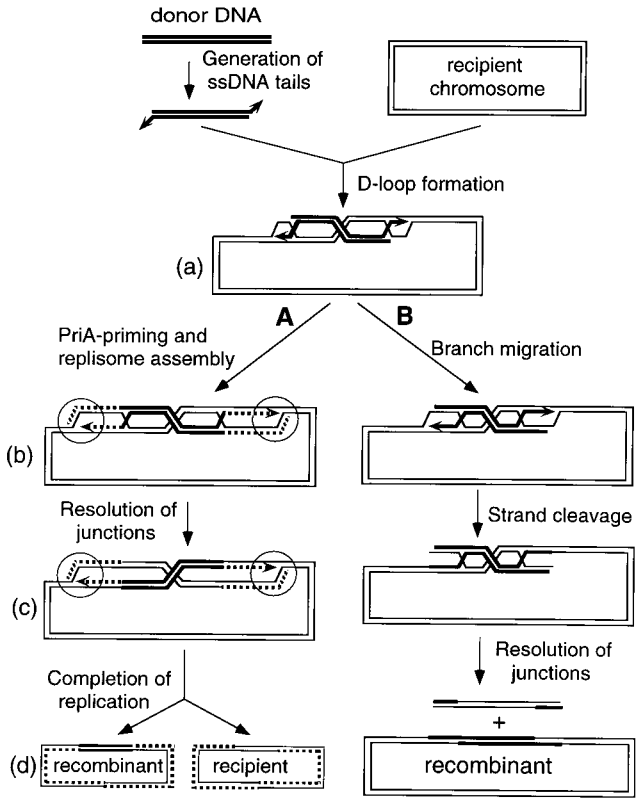

FIG. 9. Comparison of the replicative homologous recombination model (A) and a conventional recombination model (B). Donor DNA, recipient chromosome, and newly synthesized DNA are represented by thick, thin, and broken lines, respectively. Circles indicate replication forks.

This replicative mode of homologous recombination, first suggested by Smith (220) on theoretical grounds, has gained strong support from recent genetic experiments. First, the *priA* null mutation, which blocks iSDR (RDR), reduces the P1 transduction frequency by 20- to 50-fold (114, 204) and causes a significant decrease in the frequency of recombinant formation after Hfr conjugation (114). Thus, *priA* mutants, which are incapable of undergoing iSDR, are homologous recombination deficient. Second, a plasmid expressing the mutant PriA (K230R) protein, which can rectify the defect of *priA* mutants in iSDR (see the section on Gene Product Requirement, Priming Proteins, above), can also restore recombination proficiency to *priA* mutants (114, 204). Third, extragenic suppressors of the UV sensitivity of *priA* mutants can suppress the Rec$^-$ phenotype of *priA* mutants (204). All suppressor mutations map within the *dnaC* gene. As discussed above in the context of DSB repair (see the section Evidence for the RDR-Mediated DSB Repair Model), these suppressor mutations are

likely to alter the DnaC protein structure such that an active primosome can be assembled at the D-loops in the complete absence of PriA protein (204). Fourth, another mutation (*spa-47*) also suppresses the Rec$^-$ phenotype of *priA* mutants. The *spa-47* mutation maps within or very close to *dnaC* (114).

In wild-type *E. coli*, the RecBCD pathway of homologous recombination (Fig. 9) is active. *recB* and *recC* mutations inactivate the RecBCD pathway. Activation of one of the two other pathways of homologous recombination, the RecE or RecF pathway, can suppress the Rec$^-$ phenotype of *recBC* mutants (145). The RecE and RecF pathways are activated by an *sbcA* mutation and by mutations in the *sbcB* and *sbcC* genes, respectively. Thus, *recBC sbcA* and *recBC sbcB sbcC* mutants are recombination proficient. The *priA* null mutation was shown also to inhibit transductional and conjugational recombination severely in *recBC sbcA* mutants and to cause a moderate decrease in P1 transduction in *recBC sbcB sbcC* mutants (114). Therefore, the PriA protein is required not only for the RecBCD pathway but also for the RecE and RecF pathways of homologous recombination. The results suggest that the replicative homologous recombination scheme is also used in the RecE and RecF pathways. The possible involvement of extensive DNA replication in the RecF pathway has been discussed previously (110).

OTHER RECOMBINATION-DEPENDENT REPLICATION SYSTEMS

Bacteriophage T4

Bacteriophage T4 initiates DNA replication by two distinct mechanisms (reviewed in references 130, 167, and 174). Immediately after infection, T4 chromosome replication is initiated from several specific origins in a host RNA polymerase-dependent manner. When a replication fork that has started at an origin reaches the end of the T4 linear chromosome, the 3′ end of the template DNA remains unreplicated. The protruding ssDNA invades a homologous region of another molecule, or the terminal redundancy of the same molecule, to form a D-loop from which replication can be initiated. When these replication forks come to the ends of the replicating molecules, additional invasive ssDNA ends would be produced, which in turn form more D-loops for initiation. In this fashion, a complex network of highly branched and looped molecules with a number of replication forks simultaneously replicating the chromosome would result (167). The ensuing replication eventually produces large linear concatemers that can be packaged in late stages of the infection. It is clear that when a cell is multiply infected with different T4 phages, this process yields recombinants. Thus, in T4, DNA replication and recombination are intimately linked (173).

Some details of the mechanism of this RDR have been provided by in vitro studies. Formosa and Alberts (55) succeeded in reconstituting a portion of this reaction from seven purified proteins. In this reaction, UvrX protein, a RecA analog (along with the accessory protein UvrY), catalyzes the assimilation of a short ssDNA into a circular dsDNA duplex to form a D-loop in the presence of the ssDNA-binding protein, gp32. The 3′ end of the assimilated DNA serves as a primer for the leading-strand synthesis by the T4 DNA polymerase holoenzyme (gp43/gp44/gp45/gp62). Inclusion of the T4 dda helicase in the reaction mixture strongly stimulates the synthesis. Interestingly, RecA protein cannot substitute for UvrX in the replication reaction. This seven-protein reaction, however, lacks lagging-strand synthesis and yields only ssDNA copied from the template. More recently, semiconservative DNA replication was demonstrated in the reaction, which includes additional proteins. Thus, the helicase-primase complex (gp41/gp61) can be loaded into the D-loop by the action of gp59 to allow initiation of the lagging-strand synthesis (10). T4 type II topoisomerase stimulates DNA replication as the topoisomerase helps release the accumulating tension ahead of the replication fork on a covalently closed duplex template. The protein requirement for in vitro semiconservative replication initiated from a recombination intermediate closely parallels the requirement for in vivo RDR except for gp46/gp47, which is essential for in vivo RDR but is dispensable in vitro. Possible reasons for the difference have been discussed (130).

Using a plasmid model system, George and Kreuzer (65) recently demonstrated that artificially generated DSBs on a circular plasmid that carries two inverted repeats can trigger extensive DNA replication in T4-infected cells. The replication depends strictly on the products *uvrX*, *uvrY*, *32*, *46*, and *59* genes, which are also required for phage genome RDR. Analysis of the products of the in vivo reaction provided clear evidence that only one end of DSB invades the homologous region of the same molecule to form a D-loop. A replication fork assembled at the D-loop replicates the molecule to the other end. The product of the first cycle is a linear molecule of greater than unit length. The ends of the molecule have internal homology, and therefore the cycle can be repeated to generate a long concatemer (65). Naturally, this RDR reaction leads to efficient repair of DSBs, as seen in *E. coli.*

During mixed infection with T4 phage bearing an intron-containing *td* gene and T4 phage bearing an intronless allele, acquisition of the intron by the latter from the former at the precise location within the *td* gene occurs at a high frequency (reviewed in reference 33). This process, termed intron homing, has been proposed to proceed in a manner that is very similar to the process described by conventional models of DSB repair. Thus, the intronless allele is cleaved by the endonuclease I-*Tev*I (encoded by the *td* intron) to generate a DSB that is processed by degradation to yield 3′ ssDNA ends. The resulting ssDNA ends invade the homologous region (i.e., the exon) of the intron-bearing allele to form D-loops. Repair synthesis that follows successfully copies the intron sequence into the intronless allele (33). Recently, this reaction was shown to require some of the T4 gene products which are also required for T4 RDR (175). Thus, mutations in genes such as *uvrX*, *uvrY*, *32*, *43*, *41*, and *46* severely inhibit intron homing in T4-infected cells. The result suggests that the intron homing process involves RDR. Whether the DSBs generated by the intron endonuclease trigger semiconservative replication remains to be demonstrated.

Yeast

There is ample evidence that transient meiosis-specific DSBs occur at many locations in the yeast genome, which may initiate meiotic homologous recombination (18, 215, 226, 260). Whether these DSBs initiate extensive DNA replication is not known. A recent genetic study suggests that under certain conditions an artificially generated DSB triggers RDR on a yeast chromosome (155). In wild-type yeast, DSBs induced at the *MAT* locus with the HO endonuclease were efficiently repaired. This repair depends on *RAD52* but can still occur to a significant extent in the absence of *RAD51*. The analysis of the product of the *RAD51*-independent repair revealed that the repaired chromosome became homozygous for all genetic markers tested in the 100-kb region distal to the break point (155). One exciting possibility is that DSB induces semiconservative DNA replication and the replication fork copies one

arm of the chromosome to the telomere. This would be replicative homologous recombination. This replication in yeast, termed break-induced replication, is equivalent to RDR found in *E. coli* and bacteriophage T4. An interesting difference is that while prokaryotic RDR depends on RecA or its analog (UvrX), yeast break-induced replication is independent of *RAD51* (a RecA analog).

Mammalian Cells

Immunoglobulin V(D)J recombination in mice and humans is initiated by double-strand breaks, and repair of the breaks leads to recombination (86, 241). Whether this process involves DNA replication is not known. Analyses of the products of murine immunoglobulin heavy-chain switch recombination revealed point mutations, deletions, and duplications in the vicinity of the switch points (47, 48). It was suggested that the recombination events accompany local DNA synthesis, which is error prone. How widely eukaryotic cells employ RDR-mediated DSB repair and replicative homologous recombination remains to be seen.

Mitochondrial DNA

In contrast to the mitochondrial DNA (mtDNA) of mammalian cells, which exists as a monomer circle and replicates by the θ mode (34), mtDNA of most other organisms including yeast, protozoa, and higher plants exists as large concatemers often of complex structures (reviewed in reference 13). For example, newly synthesized mtDNA from tobacco cells is enriched with multigenomic concatemers of highly branched structures (183). Similar structures have also been observed in mtDNA from fungi including yeast (14). These molecules are speculated to be mtDNA replicating in a recombination-dependent manner or by the rolling-circle mode, which can be initiated by recombination (245). Similar multigenomic branched concatemers were also detected among replicating mtDNA from the malaria parasite. The possibility that these molecules replicate in a manner similar to RDR was considered (188).

CONSTITUTIVE STABLE DNA REPLICATION

SDR Mutants

As described above, iSDR is normally repressed but can be activated by SOS induction. To gain some insights into genetic bases for the activation, mutants that expressed SDR constitutively were sought. By a colony autoradiography procedure, two SDR mutants (*sdrA2* and *sdrA102*) were isolated from an *E. coli* 15 T^- strain (105, 123). Later, additional SDR mutants (e.g., *sdrA224* mutants) were isolated from an *E. coli* K12 strain (238). The mutants could continue DNA replication for at least 12 h in the presence of chloramphenicol or in the absence of required amino acids. This type of SDR in *sdrA* mutants was termed cSDR (constitutive stable DNA replication). The mutations were mapped in the *proA-metD* region of the chromosome and were recessive to the wild type.

The Idea of an Alternative Initiation Pathway

An extraordinary characteristic found with cSDR in *sdrA* mutants was that cSDR completely depends on a RecA function (123, 238). Thus, *sdrA recA*(Ts) mutants were capable of cSDR at 30°C, but the replication ceased within 2 h of a shift to 42°C with an increment of 40 to 45% in DNA content, suggesting that a RecA function was needed for initiation but not for elongation. Despite the strict requirement for RecA protein in cSDR, the growth of *sdrA* (*oriC*$^+$) mutants was not affected by *recA*(Ts), indicating the absence of a requirement for RecA protein in initiation at *oriC* in *sdrA* mutants. These observations led to the hypothesis that cSDR was initiated by a mechanism distinct from that operating at *oriC* (123). The *sdrA*$^+$ gene product was viewed as a repressor of a switch from the *oriC* initiation mechanism to the SDR initiation alternative. This switch model postulated that SOS induction inactivated the SdrA repressor, allowing the switch to iSDR, and that cSDR resulted from inactivation of the repressor by the *sdrA* mutation.

The notion that SDR initiation is distinct from and alternative to *oriC* initiation was substantiated by the demonstration that *sdrA* mutants could survive complete inactivation of *oriC* initiation (124). Thus, the *dnaA* gene could be obliterated by insertion of a Tn*10* transposon and the *oriC* site could be removed from the chromosome by deletion when cSDR was activated by an *sdrA* mutation. The resulting double mutants, *sdrA dnaA*::Tn*10* and *sdrA* Δ*oriC*, respectively, grew well, albeit slowly, in minimal media. They were, however, unable to grow in rich media such as Luria broth: the double mutants are broth sensitive.

The *sdrA* mutation can also suppress the temperature sensitivity phenotype of many *dnaA*(Ts) mutations (124). The *sdrA* mutation is therefore a non-allele-specific suppressor of *dnaA* mutations. Earlier, Atlung (9) isolated a number of thermoresistant revertants from a *dnaA46*(Ts) mutant and mapped the extragenic suppressor mutations to seven distinct loci on the chromosome. These suppressor mutations were designated *das* (DnaA suppressors). One group of *das* mutations, the *dasF* mutations, was concluded to be allelic to *sdrA* by the following criteria (237): (i) *dasF* mutations impart the cSDR phenotype; (ii) *dasF* mutants can tolerate deletion of *oriC* and complete inactivation of *dnaA*; (iii) *dasF* and *sdrA* mutants exhibit broth sensitivity; and (iv) both mutations map between *proA* and *metD*.

sdrA Mutants Are RNase HI Defective

It was known that when RNase HI was omitted from certain in vitro DNA replication systems (i.e., phage fd and plasmid ColE1), DNA replication was initiated at illegitimate sites (75, 84, 242). Thus, the presence of RNase HI ensures the initiation of DNA replication exclusively at the normal origin. This discriminatory action of RNase HI was consistent with the repressor role of $SdrA^+$ in the switch model described above. Close proximity of the map positions of *sdrA*, *dasF*, and *rnhA* (encoding RNase HI) prompted an examination of the possible identity of these genes (182). On one hand, *sdrA* and *dasF* mutants were found to be devoid of RNase HI activity. On the other hand, *rnhA* mutants were shown to have a number of phenotypes in common with *sdrA* and *dasF* mutants. For example, *rnhA* mutants exhibited cSDR, were broth sensitive, and could tolerate *dnaA*::Tn*10* and Δ*oriC*. The shared phenotypes of *sdrA*, *dasF*, and *rnhA* mutants were all complemented by a plasmid carrying a 760-bp segment of the *E. coli* chromosome containing an *rnhA*$^+$ gene (182). Thus, it was established beyond any doubt that *sdrA*, *dasF*, and *rnhA* are allelic to each other.

How could the lack of RNase HI activity result in activation of the normally repressed alternative DNA replication system which can occur in the absence of DnaA? Along the line of the switch model, it is easy to see that inactivation of RNase HI (i.e., the SdrA repressor) allows the initiation of replication at other sites as well as at *oriC*. Normally, RNase HI eliminates the RNA transcript hybridizing to the template. In its absence, the RNA is stabilized and used to initiate replication (38, 182).

Lindahl and Lindahl (142) isolated suppressor mutations, *sin*, of *dnaA46*(Ts) and found that the *sin* mutants were deficient in RNase HI activity. They proposed that RNA primers that hybridized at *oriC* for initiation of chromosome replication were substrates for RNase HI but were normally protected by DnaA protein. It followed that elimination of RNase HI rendered DnaA dispensable. This model can be formally discarded because an *rnhA* mutation renders the *oriC* site dispensable as well (124). A similar model proposed by Horiuchi et al. (80) differed from the Lindahl and Lindahl model in that it postulated the formation of RNA primers not only at *oriC* but also elsewhere. RNase HI preferentially degraded the primer at a site(s) other than *oriC* because DnaA protected the primer at *oriC*. According to the model, *oriC* might also be able to initiate without DnaA if RNase HI was inactivated, since the need for protection by DnaA was eliminated. This prediction was not borne out by subsequent experiments: *oriC* plasmids (minichromosomes) could not replicate in *rnhA dnaA*::Tn*10* double mutants (79, 121).

iSDR and cSDR Arise from Two Distinct Initiation Mechanisms

In the switch model, it was assumed that iSDR and cSDR were the same replication activity: SDR was manifested as a result of inactivation of RNase HI, either by SOS induction in iSDR or by an *rnhA* mutation in cSDR. It was predicted, therefore, that RNase HI activity should be reduced significantly during the induction. When RNase HI activity was examined during and after SOS induction by UV irradiation or nalidixic acid treatment, no reduction in RNase HI levels was found (17). There was no indication that the treatments induce an inhibitor of RNase H either. This was confirmed by in vivo experiments. It was reasoned that if the induction of iSDR resulted from the neutralization of RNase HI by an inhibitor, the presence of an excess amount of RNase HI should raise the threshold level of induction. Introduction of a plasmid overexpressing RNase HI 8- to 15-fold did not change the dose of UV needed to induce iSDR (17). Thus, neither direct nor indirect inactivation of RNase HI leads to iSDR. It is clear, therefore, that cSDR is not a constitutive expression of an inducible system, i.e., iSDR (107). As described below, comparison of the characteristics of cSDR (i.e., replication properties, origin usage, and gene product requirement) with those of iSDR (Table 1 contains a summary) clearly indicates that the two replication activities are distinct and arise from two different mechanisms of initiation.

Replication Properties of cSDR

cSDR is resistant to chloramphenicol but sensitive to rifampin (105, 246). Thus, cSDR is independent of translation but depends on transcription. cSDR initiation is inhibited during amino acid starvation in stringent (*relA*$^+$) strains but not in relaxed (*relA1*) strains. This indicates that the transcription event essential for cSDR initiation is sensitive to inhibition by ppGpp and is under stringent control (246). Consistent with this conclusion, the rate of cSDR in *rnhA* (*relA*$^+$) mutants is greatly stimulated after the addition of chloramphenicol. It is known that treatment of *relA*$^+$ strains with chloramphenicol leads to suppression of ppGpp formation and thereby to the release of stringently controlled RNA synthesis from ppGpp inhibition (29a). During cSDR, replication is semiconservative (105) and the entire chromosome is replicated (225). Unlike iSDR, cSDR is intolerant of pyrimidine dimers in templates (105) and does not seem to be error prone (112).

Origin Usage in cSDR

Although they are unable to grow in rich medium, *rnhA* Δ*oriC* double mutants manage to grow in an exponential manner in minimal medium (121). To determine whether initiation of cSDR in the double mutants occurs at a fixed site(s) on the chromosome and, if so, to locate the origin(s) on the chromosome, the marker frequency determination procedure (39) was used. This procedure gives information about the relative abundance of various sequences along the chromosome. If initiation occurs regularly at a fixed site, the relative copy number of the sequence at or near the site would be higher than those of the sequences that are replicated subsequently (39). It was found that at least five sites or regions on the chromosome, including two sites in the *terC* region, are used to initiate chromosome replication in *rnhA* Δ*oriC* double mutants (38). These origins were termed *oriK* sites (Fig. 1). Since the copy numbers of the sequences flanking a peak (*oriK*) taper off symmetrically on both sides, the replication is likely to be bidirectional. In an *rnhA oriC*$^+$ strain, both *oriK* and *oriC* sites are active. In this case, *oriC* is the dominant origin of replication (38). This observation suggested that *oriC* initiation can occur in the absence of RNase HI. Subsequent genetic analysis provided conclusive evidence for this notion (for a detailed analysis, see references 79 and 121).

Initiation at *oriK* sites alone can sustain chromosome replication to allow cells to grow in the absence of the *oriC* site at a 30 to 40% reduced growth rate and with an approximately twofold decreased DNA content (246). It was estimated that the initiation frequency of the cSDR system (per unit cell mass) is three- to fourfold lower than that of the *oriC* system. Assuming that four *oriK* sites are available and that there is no preferential use among them, it follows that the efficiency of an *oriK* as an origin of replication is 12 to 16 times lower than that of *oriC* (246). Another unique aspect of the *oriK* usage is its randomness. One origin is chosen at random from several *oriK* sites, and it fires randomly with respect to the time in the cell cycle. This random mode of *oriK* initiation is manifested in the heterogeneity seen in the cell size and DNA content of exponentially growing *rnhA dnaA*::Tn*10* double-mutant cells (246). Thus, *oriK* initiation is considerably less efficient than is *oriC* initiation and is virtually uncoupled from the cell mass.

Gene Product Requirement

The gene products known to be required for cSDR in *rnhA* mutants are listed in Table 2. cSDR is completely independent from DnaA protein but strictly dependent on RecA protein.

DnaA independence. The viability of *rnhA dnaA*::Tn*10* double mutants clearly indicates that cSDR is initiated by a mechanism independent of DnaA. In keeping with this, the *rnhA224* mutation can suppress *dnaA5*, *dnaA46*, *dnaA167*, *dnaA203*, *dnaA204*, *dnaA205*, *dnaA211*, and *dnaA508* (124). It was reported previously (105) that cSDR in a *dnaA5*(Ts) *rnhA2* (formerly *sdrA2*) double mutant was temperature sensitive and that DNA synthesis could not be seen in the presence of chloramphenicol at 42°C. Subsequent detailed studies indicated that if the temperature was raised sometime after the addition of chloramphenicol, cSDR in the *dnaA5*(Ts) *rnhA2* mutant became temperature resistant (124). The discrepancy might be due to the difference in alleles (*rnhA2* and *rnhA224*) and/or the strains used (15 T$^-$ and K-12).

RecA protein. Like iSDR, cSDR is strictly dependent on a RecA function (238). The *recA428* mutation, which specifically inactivates the recombinase activity (i.e., homologous pairing and strand exchange activity) but imparts a constitutive coprotease activity, blocks cSDR in *rnhA* mutants (113). Consistent

with this, *recA430* (formerly *lexB30*), which greatly diminishes the coprotease activity with a moderate decrease in the recombinase activity, was shown to have only a minor adverse effect on cSDR (238). Thus, the RecA function essential for cSDR is the homologous pairing and strand exchange activity.

The requirement for RecA in cSDR can be bypassed by two different mutations. The *rin* mutation suppresses the defect of *recA* mutations in cSDR (238). Thus, *rnhA* Δ*recA rin* triple mutants are capable of cSDR despite the absence of RecA function. A striking property of *rin* is that the mutation does not restore homologous recombination proficiency to *recA* mutants. This suggests that the *rin* mutation activates a function that substitutes for the RecA recombinase activity specifically in cSDR. The *rin* gene has not been cloned, and we know little about the gene product. The second type of suppressing mutation is *lexA*(Def), which inactivates the LexA repressor leading to derepression of the LexA regulon (250). Thus, cSDR can be seen in *rnhA* Δ*recA lexA*(Def) despite the complete absence of RecA activity (27, 239). It is likely that derepression of one or more of the LexA regulon genes activates a bypass pathway (the Rip pathway).

rnhA dnaA::Tn*10* and *rnhA* Δ*oriC* mutants are viable. Introduction of a *recA*(Ts) mutation into these double mutants renders the DNA replication and colony formation of these mutants temperature sensitive (121). This indicates that cSDR is the only replication system available in these mutants. To determine which stage of cSDR, initiation or elongation, was blocked by the *recA* defect, populations of the *rnhA dnaA*::Tn*10 recA*(Ts) and *rnhA* Δ*oriC recA*(Ts) mutant cells incubated at 42°C in the presence of chloramphenicol were analyzed by flow cytometry (120). It was found that after several hours of incubation, these cells contained integral numbers of chromosomes, suggesting that chromosome replication was completed without initiation of new rounds of replication in the absence of RecA function. In particular, cells of an *rnhA dnaA*::Tn*10 recA*(Ts) mutant culture that had been incubated in the absence of chloramphenicol at 42°C for several hours contained only one or two completed chromosomes. No degradation of DNA was detected in these cells during the course of incubation. These results led to the conclusion that RecA protein acts at the step of initiation in cSDR (120).

Thus, both iSDR and cSDR requires the recombinase activity of RecA protein at an initiation step. However, the similarity ends there. Whereas iSDR requires RecBCD, cSDR is not inhibited by *recB* mutations, indicating its independence from RecBCD (79). Furthermore, iSDR is mildly inhibited by *recF* and *recN* mutations and is stimulated by *recD*, *recJ*, *recG*, *ruvA*, *ruvB*, and *ruvC* mutations (see the section on Gene Product Requirement, Other *rec* Gene Products, above). In contrast, mutations in *recD*, *recJ*, *ruvA*, and *ruvC* neither inhibit nor stimulate cSDR (113). Thus, it appears that the only recombination function required for cSDR is the recombinase activity of RecA protein.

Priming proteins. cSDR requires the replicative helicase DnaB, the primase DnaG (112), and DnaC protein (105). The *priA* null mutation completely blocks cSDR, and introduction of this mutation into a strain whose survival is strictly dependent on cSDR (e.g., the *dnaA*::Tn*10 rnhA224* strain) is lethal (160). This indicates that cSDR requires PriA-catalyzed priming. It is likely that DnaB loading and subsequent priming by DnaG proceed at the initiation of cSDR in a manner similar to that found for iSDR (see the section on Priming Proteins for iSDR, above).

DNA polymerases. Like iSDR, DNA Pol III holoenzyme is responsible for the elongation stage of cSDR (Table 1). In addition, cSDR involves DNA Pol I at the initiation step (119).

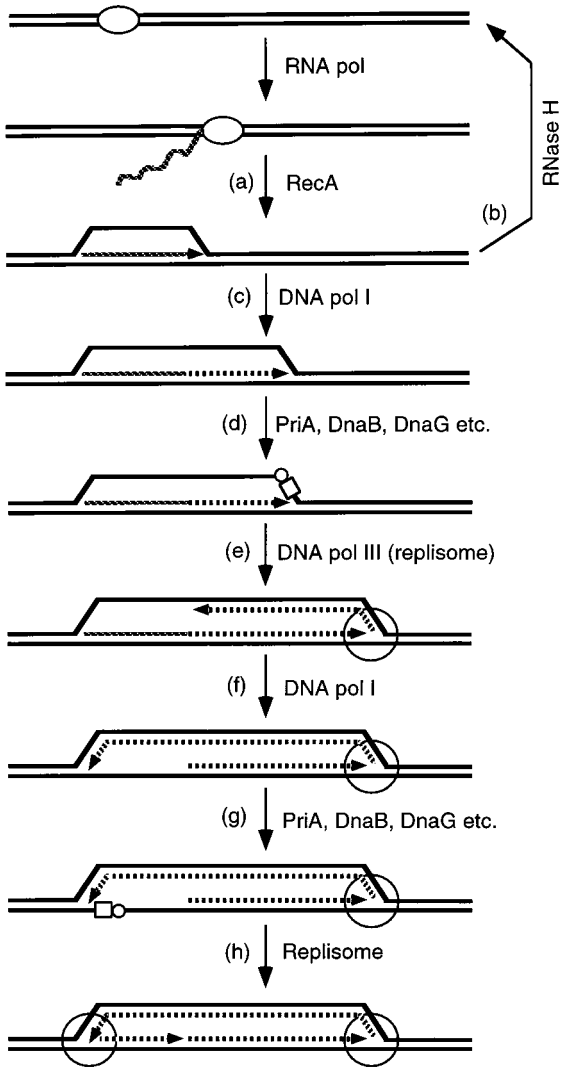

FIG. 10. Initiation of bidirectional replication at *oriK*: the R-loop model. Thick and stippled lines represent DNA and RNA strands, respectively, with small arrowheads indicating 3′ hydroxyl ends. Broken lines indicate newly synthesized DNA strands. Small ovals, squares, and circles designate RNA polymerase, DnaB, and DnaG, respectively. Large circles indicate replication forks.

Both the polymerization and 5′→3′ exonuclease activities of DNA Pol I are required. As described below, DNA Pol I is expected to play a very important role in establishing bidirectional replication at *oriK*.

R-Loop Model

As summarized above, cSDR is clearly distinguishable from iSDR in terms of gene product requirements. cSDR requires transcription and DNA Pol I but no recombination functions except for the RecA recombinase activity. Therefore, D-loop formation is not likely to be the strand-opening mechanism for cSDR. In cSDR, the initial strand opening for initiation at *oriK* is achieved most probably by hybridizing an RNA transcript to the template, displacing the other strand (Fig. 10, step a) (246). This step could involve a RecA function (see below). The resultant structure, an R-loop, is normally recognized by RNase HI and efficiently removed (step b). In *rnhA* mutants, however, the structure is stabilized and persists for a time.

DNA Pol I then synthesizes DNA from the 3′ end of the RNA, enlarging the loop (step c) (119). The PriA-catalyzed priming loads DnaB helicase, with which DnaG primase then interacts (step d). Assembly of a dimeric replisome follows, and the first lagging-strand fragment is synthesized. The newly created replication fork begins replication in one direction (step e). A DNA Pol I molecule then extends the 3′ end of the newly synthesized lagging strand, while another molecule removes the RNA with the 5′→3′ exonuclease activity (step f). Again, DnaB and DnaG are loaded on the ssDNA (step g) and replisome assembly follows. The second fork so established replicates the chromosome in the opposite direction (step h). This mechanism would achieve initiation of bidirectional replication at an *oriK* site (119).

Evidence for R-Loops

In the R-loop model, it is assumed that significantly large DNA-RNA hybrids can be generated in certain regions in the chromosome. On the other hand, in vitro studies of transcription have established that a transcript, as it is being synthesized, is expelled behind the transcribing RNA polymerase, allowing immediate rewinding of the template duplex. Thus, an actively transcribing transcription complex normally contains only a very short stretch of DNA-RNA hybrid, up to 12 bp (reviewed in reference 262), but it could be much shorter (194). Do large R-loops exist in the cell? Evidence suggests that significantly large R-loops are occasionally formed in vivo and that RNA polymerase is at least partially responsible for the formation. *rnhA* mutants were found to chronically express the SOS response, and the chronic expression is enhanced by a shift to rich medium (115). Thus, *rnhA*::*cat* null mutants have a level of SOS expression threefold higher in minimal medium and fivefold higher in Luria broth than does the wild type. It was postulated that the SOS-inducing signal derives from persisting R-loops that are formed during or after certain transcription events and stabilized in the absence of RNase HI. The formation of persisting R-loops is expected to accelerate during growth in rich medium. The displaced ssDNA in R-loops could directly act as a signal for SOS, or the persisting R-loops could block DNA replication leading to SOS induction. Importantly, mutations that enhance the chronic SOS expression in *rnhA* mutants were found among *rpoB* mutations (115). The *rpoB* gene encodes the β subunit of RNA polymerase. The finding suggested that RNA polymerase plays a role in the formation of R-loops. This notion is supported by the results of earlier in vitro experiments (30, 195).

To further analyze the possible role of RNA Pol in R-loop formation, 17 well-characterized *rpoB* mutations (89) were screened for the property that modulates the chronic SOS expression caused by an *rnhA* mutation (108). Two mutations, *rpoB2* and *rpoB3595*, were found to enhance SOS expression 2.5- and 5-fold, respectively. The enhancement could be seen only when RNase HI was absent. The *rpoB2 rnhA* and *rpoB3595 rnhA* double mutants were extremely sensitive to broth. The mutant RNA polymerases containing the RpoB3595 or RpoB2 subunit have an accelerated elongation rate during transcription in vivo and in vitro (a "fast polymerase") and are defective in Rho-dependent transcription termination (87, 88). These results suggest that R-loop formation is modulated by certain properties of RNA polymerase that also dictate the transcriptional characteristics of the enzyme. Conversely, two other mutations, *rpoB8* and *rpoB3406*, diminished SOS expression in *rnhA* mutants (108). The mutant RNA polymerases encoded by these genes are a "slow polymerase" and are termination proficient (87). Together, these results strongly suggest that RNA polymerase has a property that influences the size of R-loops, the frequency of their formation, or both. This property resides at least in part in the β subunit of the enzyme. It was previously shown that treatment with streptolydigin at a concentration that causes temporal and partial inhibition of RNA polymerase activates SDR (118). This result also points to an involvement of RNA polymerase in the activation of cSDR.

In vitro transcription of hypernegatively supercoiled pB322 results in the formation of large R-loops (45, 185). Inclusion of DNA topoisomerase I in the reaction mixture prevents R-loop formation (45). Thus, gyrase (which generates negative superhelicity) can enhance R-loop formation and topoisomerase I (which reduces it) can suppress it, at least in vitro. Recent genetic experiments provide evidence suggesting that gyrase and topoisomerase I indeed modulate R-loop formation in vivo (46). Deletion of *topA* is lethal, and Δ*topA* mutants are viable only when the cell contains a compensatory mutation in *gyrA* or *gyrB* which reduces the gyrase activity. Thus, *topA*::Tn*10* *gyrB*(Ts) double mutants are cold sensitive [and temperature sensitive due to gyrB(Ts)] because at low temperature the mutant gyrase is too active in the absence of topoisomerase I (46, 192). Importantly, overproduction of RNase HI partially suppresses the cold sensitivity of the double mutant (46). The simplest explanation for these results is that the cold sensitivity of *topA*::Tn*10* *gyrB*(Ts) stems from an increased R-loop formation stimulated by the increased activity of gyrase at low temperature because topoisomerase I, which could reduce R-loop formation, is missing. The partial suppression could come from removal of the accumulating R-loops by overproduced RNase HI. In keeping with this interpretation, a combination of a *topA* null mutation and an *rnhA* mutation is lethal even in the presence of a compensatory *gyrB* mutation (46). Also consistent is the suppression of several phenotypes (e.g., broth sensitivity) of *rnhA224* mutants by *gyrB*(Ts). As expected from the experiments described above (108), *rpoB3595* (which encodes a fast RNA polymerase) exacerbated the poor growth of *topA* mutants whereas *rpoB8* (which encodes a slow polymerase) significantly improved the growth of these mutants (46). Thus, certain properties of RNA polymerase and the state of supercoiling around the transcription unit are crucial for controlling R-loop formation.

More recently, it was demonstrated that a substantial number of R-loops can be generated when a portion (a 567-bp fragment) of the *rrnB* operon cloned in a plasmid is transcribed in vitro (186) and in vivo (163). Interestingly, the formation of R-loops depended on which strand was transcribed: R-loop formation was seen when the fragment was transcribed in its physiological (original) orientation but not in the reversed orientation. It is known that an RNA-DNA hybrid with a purine-rich RNA is more stable than a hybrid with a pyrimidine-rich RNA (198). This orientation-dependent R-loop formation may reflect the stability of RNA-DNA hybrids because transcription in the physiological orientation is expected to yield more purine-rich RNA than is transcription in the reverse orientation (163). The result suggests that the nucleotide sequence of template DNA also plays a role in R-loop formation.

RecA-Catalyzed R-Loop Formation

RecA protein acts at an initiation step of cSDR (120). This step requires the homologous pairing and strand exchange activity of RecA protein but no other recombination functions (113). The observations suggest an involvement of RecA protein with a unique capacity rather than the assimilation of ssDNA into duplex DNA to form a D-loop. This notion is also

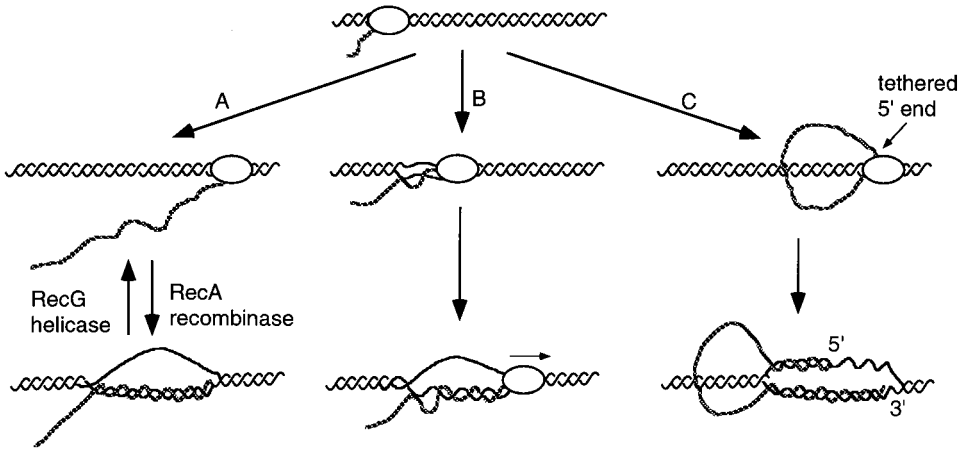

FIG. 11. Three mechanisms of R-loop formation. Solid and stippled lines indicate DNA and RNA strands, respectively. Ovals indicate RNA polymerase. See the text for details.

supported by the isolation of a mutation (*rin*) that suppresses the defect of *recA* mutations in cSDR initiation without restoring homologous recombination proficiency (238).

RecG protein is a Holliday junction-specific DNA helicase which is thought to catalyze reverse branch migration (255). The RecG helicase effectively opposes the ssDNA assimilation reaction catalyzed by RecA (254). Recent evidence (discussed below) indicates that RecG protein is capable of preventing the formation of R-loops (76) and that RecG is a junction-specific helicase that can also remove RNA from RNA-DNA hybrids (63, 244). By analogy to the demonstrated antagonistic interaction between RecA and RecG proteins in ssDNA assimilation (254), it was suggested that RecA protein could catalyze the assimilation of an ssRNA strand into the complementary region of a DNA duplex forming an R-loop and that RecG could counteract this RecA-catalyzed invasion of RNA (Fig. 11, step A) (76). Encouraged by the recent in vitro demonstration that RecA protein is capable of promoting efficient annealing (hybridization) between RNA and complementary ssDNA (100, 101), our laboratory directly tested whether RecA protein could assimilate an RNA transcript into duplex DNA in vitro (94). The results of these experiments, which will be reported elsewhere, indicated that R-loops can be efficiently formed when duplex DNA is incubated with a homologous RNA transcript and purified RecA protein in the presence of ATPγS and $MgCl_2$.

Mechanisms of R-Loop Formation

The experiments described above are consistent with the idea that in certain regions of the *E. coli* chromosome, an R-loop is occasionally formed by invasion of the duplex by a transcript that has just been synthesized by RNA polymerase and that this invasion is catalyzed by RecA protein (Fig. 11, step A). The invasion event appears to be modulated by RNA polymerase and by the supercoiling state of the region. How the RecA-catalyzed assimilation of RNA is coupled both with the transcription apparatus and with the supercoiling of the duplex is not understood. One possibility is that, simply, the way RNA polymerase transcribes, e.g., fast or slow, influences the supercoiling state of the region, which, in turn, dictates the RNA assimilation activity of RecA protein.

In two other well-studied systems, i.e., ColE1 and bacteriophage T4, R-loops are generated by different mechanisms. In ColE1 plasmid replication, a nascent transcript of the preprimer RNA II hybridizes to the template DNA strand downstream of *ori*. The hybrid (R-loop) is recognized by RNase HI and processed to generate a primer for DNA synthesis by DNA Pol I (83). It was proposed that the pairing between a stretch of 6 guanosine ribonucleotides (rG) of the nascent transcript and a stretch of 6 cytosine deoxyribonucleotides (dC) of the template DNA strand prevents rewinding of the duplex behind the transcribing RNA polymerase, allowing the formation of a persisting DNA-RNA hybrid downstream (Fig. 11, step B) (164). In the case of ColE1, therefore, R-loops are formed de novo, in contrast to the formation of an R-loop by transcript invasion in *E. coli* (Fig. 11, step A).

Immediately after infection, DNA replication in bacteriophage T4 is initiated from several fixed sites (reviewed in references 130, 172, and 174). This origin-dependent initiation involves a transcript hybridizing to the template strand, forming a persisting R-loop. The transcript is synthesized by *E. coli* RNA polymerase. A recent study analyzing the initiation events at three of the origins strongly suggests that the promoter-proximal segments (the 5′ end) of the transcript base-pair with the nontranscribed DNA strand near the transcription termination site, forming a persisting R-loop (Fig. 11, step C). These hybridizing segments can act like a wedge that facilitates access of helicases or assembly of replisomes or both (174). How the 5′-end segment of the transcript invades the duplex and how this event could assist the hybrid formation at the 3′ end are not clear. The role, if any, that *E. coli* RNA polymerase may play in this reaction is not known either.

sdrT MUTANTS

The autoradiography procedure which yielded *rnhA* (*sdrA*) mutants was also used to isolate another type of SDR mutant from *E. coli* 15 T^- (139). The mutation, *sdrT*, maps near *dnaT* and is therefore distinct from *rnhA*. Like *rnhA*, the mutation can suppress some *dnaA*(Ts) mutations such as *dnaA167* and *dnaA508* (140). Whether *sdrT* mutants tolerate *dnaA*::Tn*10* or Δ*oriC* is not known. One major difference between *rnhA* and *sdrT* is that whereas *rnhA* mutants require RecA protein only for cSDR and not for growth, the growth of *sdrT* mutants depends on RecA protein. Thus, *sdrT recA*(Ts) double mutants are temperature sensitive for growth and all DNA replication becomes temperature sensitive in the double mutants (140). This implies that initiation at *oriC* also depends on some *recA* activity in *sdrT* mutants. The reason for the RecA dependence

is not known. *sdrT* mutant cells contain as much RNase HI activity as does the wild type (17). The *sdrT* mutation appears to enhance mutagenesis with methyl methanesulfonate. This very interesting gene has not been cloned. Little is known about the SdrT protein or how the *sdrT* mutation activates cSDR.

SDR IN *recG* MUTANTS

RecG protein is a junction-specific DNA helicase that can unwind synthetic Holliday junctions (four-way junctions) and Y-junctions (three-way junctions) (147, 255). The RecG helicase is thought to oppose the assimilation reaction catalyzed by RecA protein and to catalyze reverse branch migration of Holliday junctions (254). RecG possesses the well-conserved motifs shared by the RNA/DNA helicase superfamily II (66, 146). Therefore, it could be an RNA/DNA helicase as well. As discussed below, it was recently demonstrated that simultaneous loss of RNase HI and RecG activities is lethal and that *recG* mutants exhibit a strong SDR activity (76). These results led to the conclusion that RecG protein has the capacity to compensate for the loss of RNase HI and raised the possibility that RecG protein acts as a junction-specific RNA/DNA helicase and prevents the formation of persisting R-loops (76). This was confirmed by more recent biochemical experiments (63, 244).

rnhA recG Double Mutants Are Inviable

The possibility that simultaneous inactivation of RecG and RNase HI activities is lethal was first alluded to by the failure to combine the *recG*::*kan* null with the *rnhA*::*cat* null mutation (76). This supposition was proved by the demonstration that the viability of the *recG*::*kan rnhA*::*cat* double mutant is strictly dependent on conditional expression of RNase HI in the mutant cells. Thus, the double mutant that harbored a plasmid carrying an $rnhA^+$ gene with its expression under the *lac* promoter grew well in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) but was inviable in the absence of the inducer. The inviability of the *recG*::*kan rnhA*::*cat* mutant suggested that RecG protein has an activity that promotes the removal of R-loops. A point mutation, *recG162*, blocks the branch migration function of RecG, most probably by specifically inhibiting the helicase activity of the protein (211). The *recG162* mutation was also found to severely reduce the viability of the *rnhA*::*cat* mutant (76). Thus, this function of RecG involves the helicase activity of the protein. It was suggested that RecG protein either counteracts the RecA-catalyzed invasion of a transcript into the duplex to prevent the formation of R-loops or removes RNA from the R-loop or does both (Fig. 11, step A) (76).

recG Mutants Exhibit iSDR and cSDR

If RecG prevents the formation of R-loops or removes persisting R-loops, loss of RecG activity might activate normally repressed origins of replication by a mechanism similar to that found in *rnhA* mutants. This idea was directly tested by examining *recG*::*kan* mutants to see if they undergo SDR. *recG*::*kan* mutants were found to have the capacity for SDR at a level as high as that of *rnhA224* mutants. The SDR activity in *recG*::*kan* mutants was completely dependent on $recA^+$ (76). About 50% of the SDR activity was, however, resistant to rifampin and sensitive to a *recB* mutation, indicating the presence of iSDR. This was consistent with the previous finding that the SOS response is chronically induced in *recG*::*kan* mutants due to blockade of DNA replication fork movement by unresolved recombination intermediates arising from occasional abortive recombination events during exponential growth, giving rise to iSDR (7). The remaining activity was attributed to cSDR. Thus, a *recG* mutant is a cSDR mutant. The similarity of *recG* and *rnhA* mutants is further indicated by the inviability of *recG polA* double mutants (76) and the lethality of the *rnhA polA* combination (115). ColE1-type plasmids replicating in *rnhA* mutants exist as large concatemers or multimers (224). Similarly, *recG* mutants also yield plasmid multimers (63).

Unlike *rnhA* mutants, *recG*::*kan* mutants are unable to tolerate the introduction of Δ*oriC* or *dnaA*::Tn*10* (76). The level of cSDR in *recG*::*kan* mutants may not be sufficient for survival in the absence of *oriC* initiation. Alternatively, the mode of cSDR (e.g., origin usage and the direction of replication) in *recG* mutants may not be exactly suitable to replicate the entire chromosome. The latter interpretation raises the possibility that the types of R-loops removed by RNase HI and RecG helicase are different and therefore that the origins activated are also different in the two mutants.

Resolution of R-Loops by RecG Helicase

For initiation of ColE1 plasmid replication in vitro, the preprimer RNA II must be hybridized to the template downstream of *ori* before it is processed by RNase HI to yield the primer (83). It was found that ColE1 replication is severely inhibited by the addition of purified RecG protein to the in vitro replication system (63). This replication inhibition results from dissociation of RNA II from the R-loop, which is promoted by RecG protein in an ATP-dependent manner. Similarly, purified RecG protein was shown to dissociate R-loops that were synthesized by hybridizing a 200-bp RNA molecule to a complementary sequence located within a 3-kb linear DNA duplex (244). The helicase-defective RecG162 protein was incapable of R-loop dissociation in vitro. RecG protein showed little activity to unwind a short RNA molecule hybridized to a long complementary ssDNA strand, indicating a substrate preference for a junction structure (63, 244). These in vitro results were corroborated by in vivo experiments which showed that overproduction of RecG protein drastically decreases the copy numbers of ColE1- and p15A-related plasmids (63, 244). These results led to the conclusion that RecG can act as a junction-specific RNA/DNA helicase to resolve R-loops, as suggested by the earlier genetic experiments (76).

WHAT IS cSDR FOR?

cSDR May Be a Remnant of a Primitive Replication System

cSDR has thus far been seen only in genetically altered cells, i.e., *rnhA*, *recG*, and *sdrT* mutants. It is possible, therefore, that cSDR is a genetic artifact that is fortuitously manifested by stabilization of certain R-loops, relaxing the specificity of initiation at *oriC*. The *oriK* system was speculated to be a remnant of a primitive replication system that would have operated before the *oriC* system was developed (246). Strong similarities exist between the *E. coli* RNase HI and the RNase H domains of retroviral reverse transcriptases at the primary and three-dimensional structure levels (35, 90, 125). It is tempting, therefore, to speculate that at one time both the *oriC* and *oriK* systems would have been operating in the ancestor of the present-day *E. coli* and that an *rnhA* gene might have been introduced into the cell by horizontal transfer that would have repressed the inefficient and unregulated *oriK* system, ensuring exclusive initiation at *oriC*. A similar speculation was also advanced by Wintersberger (256).

nSDR in Wild-Type Stationary-Phase Cells

Does cSDR (i.e., R-loop-dependent replication) play a physiological role? The recent finding that a cSDR-like activity can be detected in wild-type cells under specific growth conditions encourages an affirmative answer to this question. Thus, cells that are growing rapidly after a nutritional shift up acquire the ability to replicate chromosome in the presence of chloramphenicol (77). This SDR activity transiently appears when upshifted cells enter the stationary phase. Unshifted cells do not develop such an activity. Thus, activation of the SDR activity requires both nutritional shift up and entry to the stationary phase. This replication activity was hence termed nSDR (nutritional shift-up–activatable SDR) (77).

nSDR is completely dependent on a RecA function but is independent of RecB and DnaA, resembling cSDR (Table 1). However, about half of the nSDR activity seen under optimum conditions was resistant to rifampin, a characteristic of iSDR. The possibility that the rifampin-resistant part of nSDR represented iSDR was ruled out based on several criteria including the following (77). (i) A *recB* mutation does not eliminate the rifampin-resistant replication. (ii) The *lexA3*(Ind$^-$) mutation, which completely inhibits iSDR (122), does not abolish the rifampin-resistant replication. (iii) Minichromosomes, on which *oriM1* (a prominent replication origin for iSDR) resides, do not replicate under conditions in which nSDR is activated. Thus, it is possible that this part of nSDR represents a new form of SDR that is distinguishable from both iSDR and cSDR. An alternative explanation, which would make it unnecessary to invoke a third form of SDR, is to assume that nSDR is initiated from R-loops in a manner similar to cSDR and that the rifampin resistance comes from the stability of the required RNA transcripts. This explanation is supported by previous observations that cSDR in *rnhA* mutants becomes resistant to rifampin when cells are incubated with chloramphenicol for more than 1 h (105, 246).

Assuming that nSDR is a cSDR-like activity, several possible conditions resulting in the stabilization of R-loops can be considered. The first possibility is a reduction in RNase HI activity. It is known that RNase HI activity slightly decreases when SOS is induced (29, 191). It has also been suggested that heat shock proteins, DnaK, DnaJ, and GrpE, stabilize RNase HI (58). Thus, loss of the heat shock proteins could stimulate the degradation of RNase HI, resulting in activation of nSDR. Whether and how such changes in RNase HI activity are indeed manifested in rapidly growing cells upon entry into the stationary phase remain to be investigated. Preliminary assays for RNase HI in upshifted cells immediately before entry into the stationary phase in fact showed no significant decrease (78). Another possibility is that a decrease in RecG activity at the time of entry into the stationary phase triggers nSDR, but we know little about the expression of the *recG* gene or changes in RecG activity in different growth phases.

The third possibility is derepression of certain specific transcriptional units at the time of entry into the stationary phase. These transcriptional units may have a propensity to form R-loops. This suggests a coupling of nSDR activation to the expression of specific genes that is regulated by growth conditions such as nutritional shift up and growth phase. In this respect, it is interesting that mutations in the *lrp* gene, which is known to regulate a plethora of metabolic functions (180), delay the appearance of nSDR. However, an *rpoS* mutation, which inactivates the stationary-phase sigma factor, σ^s (74), affects neither the expression of cSDR nor the timing of it (77). The fourth possibility is a change in the properties of RNA polymerase that would result in increased production of R-loops usable for initiation of nSDR. Certain changes in RNA polymerase properties during the stationary phase have been reported (184). Whether any of these mechanisms or perhaps others activate nSDR remains to be seen.

Possible Physiological Role of cSDR

The *oriK* system is an inefficient and virtually unregulated replication system (246). This is a stark contrast to the extremely well-regulated and efficient *oriC* system (247). Owing to the *oriC* system, *E. coli* can respond to rapid changes in growth conditions and precisely adjust the initiation frequency to a wide range of growth rates within the laboratory conditions. The life of *E. coli* includes an easy life within a human intestine and a hard life outside the body. I wish to suggest that the *oriC* system is designed for the time of rapid and slow growth while *E. coli* enjoys nutritionally rich environments and that the *oriK* system is designed for life in starving environments. It is imaginable that during nutritional stress, cells require a very limited amount of DNA replication, which is not necessarily regulated precisely. It would be advantageous for the resting cells to be able to continue initiating replication without additional protein synthesis, however inefficient the process would be. During the transition from the rich environment to the poor one, cSDR could be activated as demonstrated for nSDR upon the transition from the active growth phase to the stationary phase. It should be pointed out that although the appearance of nSDR is transient, i.e., a peak at the time of entry into the stationary phase followed by a rapid decline (77), the capacity for nSDR could persist, although measurable nSDR decreases considerably due to limited amounts of precursors and energy in the stationary phase. It is likely that the capacity for the opportunistic initiation at *oriK* sites could persist during the life of *E. coli* in the starving environments. Only upon reentry to the human intestine is the *oriC* system activated and the cells prepared for rapid growth. Simultaneously, the conditions that prevent the formation of persisting R-loops returns, ensuring exclusive initiation at *oriC*, which is essential for well-controlled rapid growth.

cSDR as a Research Tool

Clearly, cSDR in *rnhA* mutants is capable of sustaining chromosome replication in the complete absence of normal replication originating from *oriC*. This unique property of cSDR activated in *rnhA* mutants has been exploited on several occasions to determine whether certain mutations or conditions specifically affect *oriC* initiation. One example involves the experiments to determine if a particular mutant *oriC* is inactive in a cell that lacks the DNA-binding protein FIS (11). One of the DnaA boxes (R4), which had been believed to be essential for the *oriC* function, was recently demonstrated to be dispensable: the DnaA box could be deleted from the *oriC* site in the chromosome without causing a loss of viability (11). This deletion (*oriC207*), however, could not be introduced by P1 transduction into a *fis* mutant which lacks FIS protein. This suggested that a combination of *oriC207* and *fis* is lethal because the two mutations together cripple the *oriC* function. To prove this supposition, an *rnhA fis* double mutant was used as a recipient of P1 transduction. The *oriC207* deletion was successfully introduced into this strain, owing to the activated cSDR. Introduction of a plasmid carrying an *rnhA*$^+$ gene rendered the *rnhA oriC207 fis* triple mutant inviable (11). These results indicated that the *oriC207* and *fis* mutations can be combined only when cSDR is active. It was concluded that *oriC* initiation is inoperative in the *oriC207 fis* double mutant.

Another example of the use of *rnhA* mutants as a research

tool involves the experiments to determine whether anionic phospholipids are essential for initiation at *oriC* in vivo (261). In vitro evidence indicates that anionic phospholipids can rejuvenate DnaA protein by converting the inactive DnaA-ADP form to the active DnaA-ATP form, suggesting a crucial role of the lipids in *oriC* initiation. The difficulty in demonstrating the requirement of anionic phospholipids for *oriC* initiation in vivo is that the lipids are also essential for other cellular processes. Xia and Dowhan (261), by genetically manipulating the synthesis of anionic phospholipids, found a concentration of the lipids that allows *rnhA* mutants but not *rnhA*$^+$ cells to survive. Certain conditions (e.g., introduction of a *recA* mutation) that turn off cSDR in the mutant cells rendered the cells inviable. Thus, at this concentration of the lipids, the *oriC* system is inoperative and the cells are viable only because cSDR is activated. These results permitted the investigators to conclude that anionic phospholipids are essential for initiation at *oriC*.

Besides the roles that DnaA protein plays in *oriC* initiation, the protein is also involved in the repression or activation of certain genes and in transcription termination (169). A recent study also suggested that DnaA protein binds DNA in a sequence-independent manner and modulates the supercoiling of the chromosome (170). The viability of *rnhA dnaA*::Tn*10* double mutants (124) indicates that DnaA is essential only for initiation at *oriC* and has no essential function in any other cellular processes. It can be concluded, therefore, that the roles of DnaA in these other activities are only minor and cannot be essential ones.

CONCLUDING REMARKS

Is DnaA Protein the Unstable Factor?

The studies of SDR began with the aim to find the reason why concomitant protein synthesis is required for continued initiation of chromosome replication (117). Over a quarter of a century later, the answer to this question is still elusive. A most straightforward hypothesis is that a factor(s) that is essential for initiation at *oriC* is unstable and must be constantly resynthesized for initiation. The best candidate for such a factor was DnaA protein, which is specifically required for *oriC* initiation. It was found, however, that the cellular amount and synthesis of DnaA protein are constant throughout the cell cycle and that the protein is very stable during exponential growth (203). Thus, the unstable DnaA protein hypothesis had to be abandoned. More recent experiments, however, seem to encourage the revival of this idea. The *dnaA*(Cos) mutation was isolated as a suppressor of the *dnaA46*(Ts) mutation (97). *dnaA*(Cos) mutants are cold sensitive and overinitiate chromosome replication at low temperatures. Intriguingly, initiation in *dnaA*(Cos) mutants can continue for several hours in the presence of chloramphenicol whereas *dnaA*$^+$ cells cease replication within 1 h (97), suggesting that the DnaA(Cos) protein activity is considerably more stable than the activity of DnaA$^+$. This characteristic of the DnaA protein was also seen in a crude extract system for *oriC* initiation: wild-type DnaA protein supports minichromosome replication only for a limited period (15 min), whereas the same amount of DnaA(Cos) protein can continue initiating for at least 45 min (95). Thus, the wild-type DnaA activity has a limited longevity during the incubation with crude extract. This suggested that wild-type DnaA protein might be inactivated by a factor in crude extracts and that DnaA(Cos) protein might be insensitive to the factor. Further studies revealed that an activity in crude extracts indeed specifically inactivates DnaA protein in a manner dependent on ATP hydrolysis (96). No significant change in either the amount of DnaA protein or its apparent molecular mass during incubation with crude extract was detected by immunoblot analysis. This finding is consistent with the earlier in vivo results which showed considerable stability of DnaA protein (203). As expected, DnaA(Cos) protein was resistant to the inactivating factor (96). The finding raises the possibility that DnaA protein must be resynthesized for continuous initiation. This hypothesis, however, requires that the inactivation reaction be irreversible such that the protein is no longer reusable. Whether the inactivation factor does inactivate DnaA protein irreversibly is not known.

SDR Studies

Although these studies have not provided a definitive answer to the original question, i.e., why *oriC* initiation requires concomitant protein synthesis, the investigation on SDR has yielded a wealth of information about chromosome replication systems in *E. coli*. The studies introduced, for the first time, the idea of alternative initiation mechanisms that could operate in *E. coli*. The work on iSDR has revealed that after SOS induction, the modes of chromosome replication are drastically altered. The iSDR studies have helped to expand our understanding of the homologous recombination and DSB repair processes which could involve extensive semiconservative DNA replication to an extent that was not seen before. It is surprising that *E. coli* cells normally possess such a capacity and exercise it in a controlled manner. This feature is particularly remarkable when viewed in light of the suggestion that similar recombination-dependent replication found in phage T4 is designed to rapidly accelerate DNA synthesis after infection and to escape the host control mechanism which coordinates DNA replication and cell division (172).

The studies of cSDR have clearly shown that cells can activate a normally repressed replication system that could allow them to survive in the complete absence of the major initiation system, the *oriC* system. By elucidating the poorly functioning and unregulated *oriK* system, the studies have helped underscore the extraordinary precision and efficiency that the *oriC* system can achieve in the initiation of chromosome replication. The cSDR studies have also helped us realize that certain transcriptional events can yield R-loops that contain a large DNA-RNA hybrid and have revealed the conditions and factors that affect R-loop formation, such as the supercoiling state of the chromosome, RNA polymerase, RNase HI, and RecG protein.

The structure of *oriC* (111) and the amino acid sequence of DnaA protein (218) are well conserved among a number of eubacteria, suggesting that the initiation mechanism for chromosome replication in eubacteria has been largely conserved in evolution. Whether other eubacteria besides *E. coli* possess activities similar to iSDR and cSDR remains to be explored.

Fundamental cellular processes such as chromosome replication, homologous recombination, DNA damage repair, and transcription are each complex. Consequently, these processes have been traditionally studied separately. It is obvious that these processes do not operate independently from each other in the cell: certain degrees of interaction are naturally expected. The SDR systems have provided opportunities to explore such interactions, and the studies have revealed parts of the intricate interplay between DNA replication and homologous recombination and between replication initiation and transcription. It has been argued that initiation at *oriC* requires a transcription event (reviewed in references 168 and 218). A recent study has indicated, however, that this transcriptional activation can be seen only when chromosomal *oriC* is under

suboptimal conditions (12). Nevertheless, transcriptional activation represents another link between replication initiation and transcription (73a). Studies by others have uncovered a link between transcription and DNA damage repair (70, 210) and have elucidated a mechanism of DNA replication-dependent transcription in bacteriophage T4-infected cells (23). It is hoped that a more broadly integrated picture, a network, of interacting cellular activities will emerge in the near future. The SDR systems will continue to provide a basis for understanding the complex interactions between various cellular functions.

The approach used for the SDR studies has thus far been primarily genetic and physiological. Without doubt, better understanding of the interdependence of these cellular activities demands the development of in vitro systems followed by biochemical analysis. Nevertheless, the SDR studies have demonstrated the analytical and synthetic power of the integrative physiology-genetics approach to a complex biological problem.

ACKNOWLEDGMENTS

I thank Tsuneaki Asai, Dave Bear, Erik Boye, Steve Kowalczykowski, and Hideo Shinagawa for critically reading the manuscript and for offering comments to improve it. I also thank Tsuneaki Asai for numerous discussions on some of the models that are presented in this article. I am particularly grateful to the following individuals who provided me with friendship and encouragement in the early years of the investigation when the SDR phenomenon was ignored by many: Dave Bear, Erik Boye, Dick D'Ari, Olivier Fayet, Walter Messer, Gisela Mosig, Kirsten Skarstad, Kaspar von Meyenburg, and Evelyn Witkin. I sincerely hope that the contributions by a number of individuals who worked, with unabated enthusiasm and diligence, in my laboratory during the past 23 years are well documented in this article. I am indebted to Fusae Kogoma and Takeshi Kogoma for their patience and understanding, and I dedicate this article to them.

The work from my laboratory was supported by Public Health Service grant GM22092 from the National Institutes of Health and by grant BIR-9218818 from the National Science Foundation.

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